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Project Title: Relationship of the genus *Savoryella* (teleomorph ascomycete) and its anamorph *Canalisporium*, as inferred by multiple gene phylogenies

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SUMMARY

The taxonomic placement of freshwater and marine *Savoryella* species has been widely debated in many respects and their anamorphs have never been reported. This study incorporates individual phylogenetic datasets and a combined dataset, based on the small subunit rDNA (SSU), large subunit rDNA (LSU), to determine the ordinal position of the genera *Ascotaiwania*, *Canalisporium* and *Savoryella*, all based on strains isolated from Thai substrata. Other genes sequenced include LSU rDNA, ITS region, RNA polymerase II the second largest subunit (RPB2).

In this study, the ascomycete *Ascotaiwania* which is morphologically similar to *Savoryella*, was included in the study. *Ascotaiwania* is characterized by ascospores that are generally more than 3-septate with hyaline end cells, asci with a relatively massive, and a non-amyloid apical ring. The ordinal status of these two genera is unknown and consequently classified as Ascomycota *incertae sedis*.

We also studied selected species of the anamorphic genera: *Monotosporella, Helicoon* (anamorphs of the genus *Ascotaiwania*), and *Canalisporium* species (Thai isolates).

Phylogenetic analyses indicate that the genera *Savoryella, Ascotaiwania* and *Canalisporium* share a common ancestor and are closly related. In the SSU rDNA, LSU rDNA and RPB2 dataset, *Savoryella* shows no affinities with the Hypocreales, Halosphaeriales, Sordariales and Xylariales (subclass Hypocreomycetidae, Sordariomycetes) despite earlier assignment to the order (Sordariales, Hyprocreles). These findings suggest a new lineage of aquatic ascomycetes that have invaded both the marine and freshwater environments. Although these genera are related, tree topologies between the differenet datasets vary as they contain differenet taxa. However, they form a distinct group similar to the unclassified group of marine ascomycetes comprising *Swampomyces, Torpedospora* and *Juncigera* (Schoch et al 2007).

However a number of trends can be discerned:

1. *Savoryella* species form a monophyletic clade, although the marine and freshwtaer species are placed in different sister groups.
2. A new ascomycete, showing similarities to *Ascotaiwania*, groups in all anlayses with *Canalisporium* speices and may be a new genus.

3. *Ascotaiwnaia* is not monophyletic with the differnet species grouping with differenet anamorphs.

CONTENT

CONTENT (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

PART I

GENERAL INFORMATION

PART I

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Project Title: Relationship of the genus *Savoryella* (teleomorph ascomycete) and its anamorph *Canalisporium,* as inferred by multiple gene phylogeny

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PART II

PROGRESS REPORT (1 YEAR)

PART II

BACKROUND AND RATIONAL

An investigation of the fungal diversity of Thailand known as "*lignicolous freshwater fungi*" has been in progress for 8 years with over 400 species from various locations and wood species documented (Sivichai, 1999; Sivichai et al., 2002; Sivichai et al., 2003; Sivichai and Boonyene, 2004). The majority of the water-dwelling fungi recorded in this study were mitosporic fungi, with few Ascomycota and only one Basidiomycota (Sivichai, 1999; Sivichai and Jones, 2004).

Savoryella is one of the most commonly reported unitunicate ascomycete genus from submerged wood in rivers or streams (Sivichai et al., 2002, 2003). The phylogenetic assignment of the genus is unresolved and it has been referred to a number of orders and families in the Sordariomycetes, Sordariomycetidae (Zhang et al., 2006) as shown the following Table 1.

Table 1. Taxonomic assignment of the genus *Savoryella*

Authors & References	Order	Family	Comments
Jones & Eaton 1969	_	_	Authors did not assign to any family
Kohlmeyer & Kohlmeyer, 1979	Sphaeriales *incertae sedis*	_	_
Kohlmeyer, 1986 Eriksson & Hawksworth, 1986	Ascomcyetes *incertae sedis*	_	_
Eriksson & Hawksworth, 1987	Xylariales	Amphisphaeriaceae	_
Jones & Hyde 1992	Sordariales	Tripterosporaceae, Lasiosphaeriaceae	Presence of brown ascospores , asci with a refractive apical ring
Barr, 1990	Halosphaeriales	_	Presence of catenophyses-like paraphyeses
Vijaykrishna, 2005 Tsui & Hyde, 2003	Hypocreales	_	Based on molecular analysis

Jones and Eaton (1969) first described this genus with black perithecial ascomata, cylindrical asci with a comparatively flattened apical ring and brown ascospores with hyaline end-cells. It was collected on wooden slats in a water cooling tower run with brackish water at Connah's Quay, North Wales. Currently 11 species

are recognized of which three are marine, one occurs on wood associated with sand, while the remainder are found in freshwater habitats.

No anamorph has been reported for *Savoryella* (Tsui and Hyde, 2003). The establishment of the anamorph-teleomorph link between taxa can be phylogenetically informative. Of the 400 freshwater species documented only 56 links have been established between ascomcyetes and anamorphic fungi (Sivichai and Jones, 2003). Of 22 anamorph/teleomorph connections reported by Sivichai (1999), most of these were detected by observing the anamorph/teleomorph growing together on the same substratum and then verifying the connections by cultural studies. Recently, Sivichai (personal observation) collected an *Ascotaiwania* (*Savoryella*)-like species growing on submerged wood in (Khlong I Gading stream, Halabala wildlife sanctuary, Narathiwat, Thailand) that produced a *Canalisporium* species in culture.

The anamorphic genus *Canalisporium* is characterized by possession of a dolipore-like septum at the transmission electron microscope level, but no teleomorph is known for the genus (Ho, 1999; Goh et al., 1989; Nawawi and Kuthubutheen, 1989).

The genus *Ascotaiwania* is reported from freshwater habiata and from terrestrial palms with 12 species.

Presently, one gene approach has been used for studying the relationships between fungi, but it may not infer the whole evolution of fungal taxa as different genes evolve at different rates (Li and Graur, 1991; Geiser et al., 2000). Molecular phylogeny techniques on nuclear ribosomal genes (LSU, SSU, 5.8S rDNA) and the mitochondrial gene ß-tubulin gene offer the chance to investigate the taxonomic placement and sexual/asexual relationships of a wide range of fungal taxa. Other genes that can also enhance our knowledge of fungal evolution include: RNA polymerase II subunit (Kurtzman and Robnett, 2003), and translation elongation factor EF1-α (O'Donnell, 2000; Kurtzman and Robnett, 2003).

OBJECTIVES

1. To determine the taxonomic placement of *Savoryella* by multiple gene phylogeny.

2 To elucidate the phylogeny of *Savoryella* with other phenotypically similar genera

3. To examine the interrelationships of the genera *Savoryella* and *Ascotawania* with the anamophic genus *Canalisporium* from different habitats (freshwater and marine environments) based on morphological and molecular data

SCOPE OF THE STUDY

This work was focused on the molecular analysis of the ribosomal DNA gene: small subunit (18S), large subunit (28S), internal transcription spacers (ITS) and Rpb2 gene from those cultures (table 2) in order to clarify or better classify them at the family and ordinal level, where problems have been encountered in the delineation of genera using traditional taxonomic characters.

MATERIAL AND METHODS

1. Specimen collection and fungal growth

Fungi were isolated from various substrata from freshwater and marine locations in Thailand (Sivichai and Boonyene, 2004; Sakayaroj et al., 2005; Pinruan et al., 2002) and maintained on CMA or PDA media with seawater or freshwater (Table 2). All cultures were grown on potato dextrose agar (PDA) at room temperature of 25°C for 4-16 weeks (depending on the growth rate of each species).

2. Genomic DNA extraction

Actively growing mycelia were scraped off the surface of a culture and transferred to micro-centrifuge tubes and the biomass lyophilized at -80°C for 2 days before DNA extraction which followed a modified protocol of Tigano-Milani et al. (1995). The lyophilized-mycelia were ground with a sterile pipette tip in 2 ml microcentrifuge tube. The resulting powder was transferred to a 1.5-mL pre-warmed (65°C) microcentrifuge tube with 700 µl extraction buffer (0.7 M NaCl; 50 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; 1% CTAB) and incubated at 65°C for 1 hour. In the CTAB-based method, DNA was extracted once with 500 µl (24:1) chloroform-isoamyl alcohol (CIAA) and centrifuged at 12.000 rpm for 20 minutes. The supernatant was transferred to a 1.5-mL new microcentrifuge tube containing 1/10 volume of 10% CTAB, added with 700 µl CIAA and centrifuged for 20 minutes at 12.000 rpm. The 1000 µl precipitation buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% CTAB) were added to the aqueous phase of supernatant for 30 minutes at room temperature. The 300 µl Tris-EDTA High Salt (1 M NaCl; 10 mM EDTA, pH 8.0; 1 mM EDTA, pH 8) buffer were added to the pellet, washed with 400 µl ethanol 70%, and resuspended in 30 µL sterilized deionized water containing 5 µ RNase A (100 µg/mL). The DNA pellet after centrifugation (20 minutes, 12.000 rpm, 4 °C) was washed in 400 µl 70% ethanol and air-dried. Finally, the DNA re-suspended in 50 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0).

3. PCR amplification

DNA was amplified with Taq DNA polymerase. Different regions of the partial SSU, LSU ribosomal DNA, ITS region (Figure 1) and partial RPB2 (Figure 2) were amplified using primers (Table 3) NS1, NS3, NS4, NS5, NS6, JS1, JS8, LROR, LR5, LR7, ITS1, ITS4, ITS5, RPB2-5F2 and RPB2-7CR (White et al., 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al., 1999) using PCR Model MJ Research DYAD ALD and PCR reaction were carried out in total volume of 50 µl containing 10-50 ng DNA template. The 10X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs, 0.2 µM each primer and 0.5 U of Taq Polymerase (DNA Polymerase Kit, Vivantis Technologies). Amplification cycles were performed following the procedure of Tang et al. (2007) composed of 95°C for 5 min, followed by denaturation step at 35 cycles, 52°C for 1 min (for SSU or LSU rDNA), 55°C for 1.5 minute (ITS region), 55°C for 1.5 minute (for RPB2) at annealing step, 72°C for 1.5 minutes (elongation step) and the final step of 72°C for 10 minutes. The size of each amplified fragment was verified by gel electrophoresis with ethidium bromide staining of a 2 mL product sample and visualized over an ultraviolet transilluminator. PCR products were purified using NucleoSpin^R Extract Kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Then checking for the quantity and quality in a 1% agarose gel electrophoresis was applied. Finally, the purified PCR product was used directly for DNAsequencing (Table 4-5).

Table 2. Fungal isolates sequenced for this study

Species	Isolates numbers	Sources	Substrate origins/Habitats	Collection sites	Fungal references	GenBank accession numbers			
						SSU	LSU	ITS	RP B2
Ascotaiwania sawadae	SS00051	BCC03343	Submerged Hard wood/Freshwater	Khao Yai National Park, Nakhon Nayok, Thailand	H.S. Chang & S.Y. Hsieh (1998)	N/A	N/A	N/A	N/A
Ascotaiwania-like sp. nov	SS03615	BCC20507	Submerged *Wrightia tomentosa*/Freshwater	Khlong I-Gading stream, Hala-Bala Wildlife Sanctuary, Narathiwat, Thailand	N/A	N/A	N/A	N/A	N/A
Canalisporium sp.(*caribense*)	SS03732	BCC21424	Submerged wood/Freshwater	Stream at Ban Krang, Kaeng Krachan National Park, Phetchaburi, Thailand	N/A	N/A	N/A	N/A	N/A
Canalisporium caribense	SS03683	BCC21022	Submerged wood/Freshwater	Wang Kar Leung Waterfall, Wang Kan Lueng Arboretum, Lop Buri, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium caribense	SS03839	BCC24239	Submerged wood/Freshwater	Khlong I-Gading stream, Hala-Bala Wildlife Sanctuary, Narathiwat, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium elegans	SS00523	BCC003625	Submerged *Xylia*	Stream at road marker at km 29.2,	Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A

			dolabriformis/Freshwater	Khao Yai National Park, Nakhon Ratchasima, Thailand					
Canalisporium elegans	SS00877	BCC09963	Submerged wood/Freshwater	Stream at road marker at km 18, Kaeng Krachan National Park, Phetchaburi, Thailand	Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium elegans	SS00895	BCC12772	Submerged *Stereospermum neuranthum* /Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium elegans	SS03483	BCC26225	Submerged wood/Freshwater	Bor Kleng Hot Spring, Ratchaburi, Thailand	Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium elegans	SS03491	BCC18364	Submerged wood/Freshwater	Kaeng Krachan National Park, Phetchaburi, Thailand	Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium exiguum	SS00809	BCC12770	Submerged wood/Freshwater	Khao Soi Dao Wildlife Sanctuary, Chanthaburi, Thailand	Goh & K.D. Hyde (1998)	N/A	N/A	-	N/A
Canalisporium pallidium	SS00091	BCC03350	Submerged *Alstonia scholaris*/Freshwater	Streams at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	Goh, W.H. Ho & K.D. Hyde (1998)	N/A	N/A	N/A	N/A
Canalisporium palladium	SS00498	BCC03608	Submerged *Xylia dolabriformis*/Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon	Goh, W.H. Ho & K.D. Hyde (1998)	N/A	N/A	N/A	N/A

				Ratchasima, Thailand					
Canalisporium pulchrum	SS00170	BCC03406	Submerged *Alstonia scholaris*/Freshwater	Stream at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium pulchrum	SS03773	BCC21030	Submerged Leaf/Freshwater	Khlong I-Gading Stream, Hala-Bala Wildlife Sanctuary, Narathiwat, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium pulchrum	SS03788	BCC22507	Submerged wood/Freshwater	Khao Pra - Bang Khram Wildlife Sanctuary, Krabi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	-	N/A
Canalisporium pulchrum	SS03819	BCC21221	Submerged wood/Freshwater	Khao Pra-Bang Khram Wildlife Sanctuary, Krabi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Canalisporium pulchrum	SS03823	BCC21428	Submerged wood/Freshwater	Khao Pra-Bang Khram Wildlife Sanctuary, Krabi, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	-	-
Canalisporium pulchrum	SS03982	BCC23549	Submerged wood/Freshwater	Haew Narok waterfall, Khao Yai National Park, Nakhon Nayok, Thailand	(Hol.-Jech. & Mercado) Nawawi & Kuthub. (1989)	N/A	N/A	N/A	N/A
Savoryealla aquatica	SS00096	BCC03345	Submerged *Anisoptera oblongal*/Freshwater	Streams at road marker at km 29.2, Khao Yai National Park, Nakhon Ratchasima, Thailand	K.D. Hyde (1993)	N/A	N/A	N/A	-

Savoryella aquatica	SS00359	BCC03521	Submerged *Alstonia scholaris*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	K.D. Hyde (1993)	N/A	N/A	N/A	-
Savoryella aquatica	SS00583	BCC03641	Submerged *Xylia dolabriformis*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	K.D. Hyde (1993)	-	N/A	N/A	-
Savoryella aquatica	SS03801	BCC22509	Submerged wood/Freshwater	Khao Pra - Bang Khram Wildlife Sanctuary, Krabi, Thailand	K.D. Hyde (1993)	N/A	N/A	N/A	-
Savoryella lignicola	SAT**00908**	-	-	Tammarang Pier, Saton, Thailand	E.B.G. Jones & R.A. Eaton (1969)	N/A	N/A	N/A	N/A
Savoryella longispora	SAT00320	BCC23612	Mangrove wood/Marine	Tammarang Pier, Satun, Thailand	E.B.G. Jones & R.A. Eaton (1969)	N/A	N/A	N/A	N/A
Savoryella longispora	SAT00322	BCC23592	Mangrove wood/Marine	Tammarang Pier, Satun, Thailand	E.B.G. Jones & R.A. Eaton (1969)	N/A	N/A	-	-
Savoryella paucispora	SAT00866	BCC28374	Mangrove wood/Marine	Laem TaLum Phuk, Nakhonsithammarat, Thailand	(Cribb & J.W. Cribb) J. Koch (1982)	N/A	N/A	N/A	N/A
Savoryella paucispora	SAT00867	BCC28375	Mangrove wood/Marine	Laem TaLum Phuk, Nakhonsithammarat, Thailand	(Cribb & J.W. Cribb) J. Koch (1982)	N/A	N/A	N/A	N/A
Savoryella verrucosa	SS00042	BCC03342	Submerged Elephant grass/Freshwater	Khao Yai National Park, Nakhon	Minoura & T. Muroi	N/A	N/A	N/A	N/A

				Ratchasima, Thailand	(1978)				
Savoryella verrucosa	SS00052	BCC03344	Submerged Twig/Freshwater	Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)	N/A	N/A	N/A	N/A
Savoryella verrucosa	SS00582	BCC03642	Submerged *Xylia dolabriformis*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)	N/A	N/A	N/A	N/A
Savoryella verrucosa	SS03331	BCC24236	Submerged *Stereospermum neuranthum*/Freshwater	Streams at Tad Tha Phu, Khao Yai National Park, Nakhon Ratchasima, Thailand	Minoura & T. Muroi (1978)	N/A	N/A	N/A	N/A

a Isolates with the prefix SS and SAT are from the BIOTEC Culture Collection (BCC);

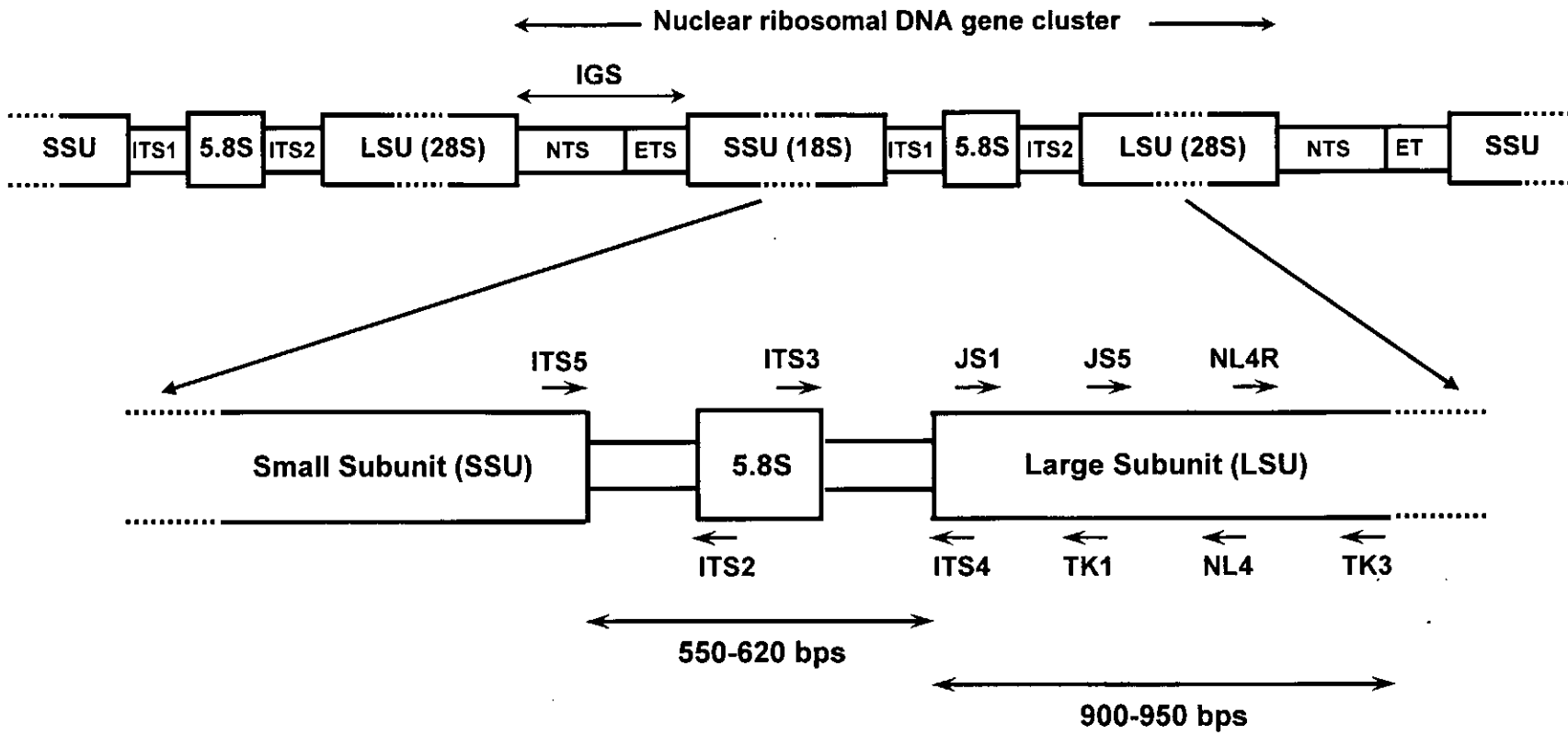

Figure 1. Diagrammatic representation of the nuclear ribosomal DNA gene cluster showing the primer positions for the PCR and DNA sequencing. The gene is divided into coding (SSU, 5.8S and LSU genes) and non-coding (IGS and ITS) regions. Position and direction of replication of each primer are shown. Picture from Kwong, 2003

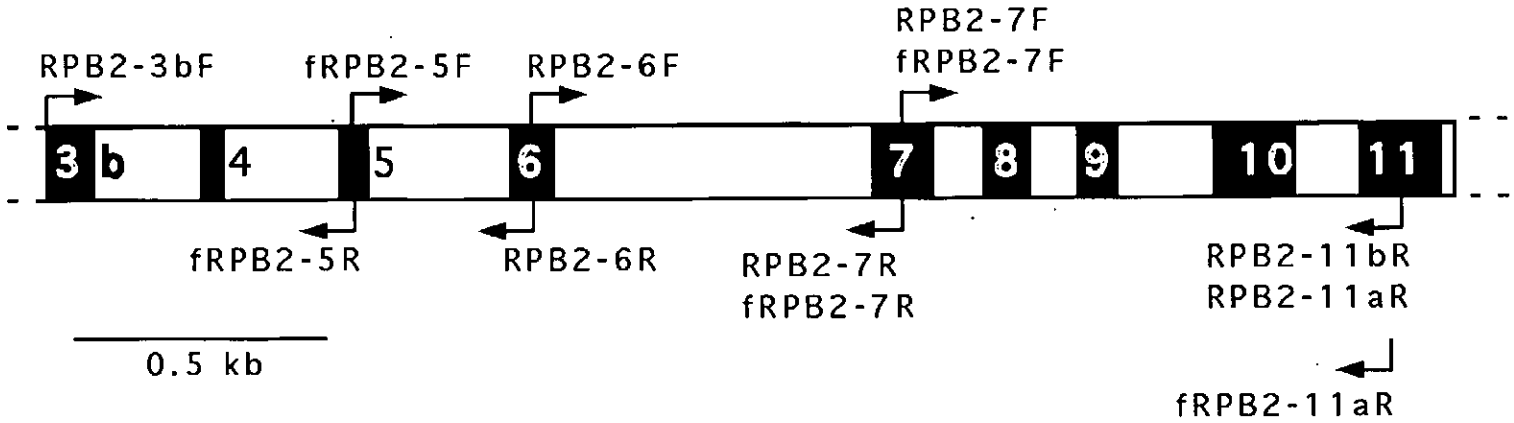

Figure 2. Diagrammatic representation of the RNA polymerase II gene (RPB2) encoding the second largest protein subunit showing the primer positions for the PCR and DNA sequencing. Blocks with shading are exons (coding regions) and blocks without shading are introns (non-coding regions). Picture from http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.htm

Table 3. Primers used for PCR and DNA sequencing

Primers	Sequence (5' – 3')
Small subunit (18s)	
NS1	GTA GTC ATA TGC TTG TCT C
NS3	GCA AGT CTG GTG CCA GCA GCC
NS4	CTT CCG TCA ATT CCT TTA AG
NS5	AAC TTA AAG GAA TTG ACG GAA G
NS6	GCA TCA CAG ACC TGT TAT TGC CTC
Large subunit (28s)	**Sequence (5' – 3')**
JS1	CGC TGA ACT TAA GCA TAT
JS8	CAT CCA TTT TCA GGG CTA
LR5	
LR7	TAC TAC CAC CAA GAT CT
LROR	ACC CGC TGA ACT TAA GC
Internal Transcribed Spacers (ITS)	**Sequence (5' – 3')**
ITS1	TCC GTA GGT GAA CCT GCG G
ITS4	TCC TCC GCT TAT TGA TAT GC
ITS5	GGA AGT AAA AGT CGT AAC AAG G
Polymerase II second largest subunit regions 5-7 (RPB2)	**Sequence (5' – 3')**
RPB2-7cR	CCC ATR GCT TGT YYR CCC AT
RPB2-5F2	GGG GWG AYC AGA AGA AGG C

Table 4. A master mix prepared for each PCR reaction

Reagents	Volume added	Final concentration
10X PCR buffer with $MgCl_2$	5.0 µl	1 X
10 mM dNTPs mix	1.0 µl	1.5 mM
10 mM forward primer	1.0 µl	0.2 µM
10 mM reward primer	1.0 µl	0.2 µM
Taq DNA polymerase (Enzyme)	0.5 µl	0.5 unit
Genomic DNA	2.0 µl	10-50 ng
Sterile H_2O	39.5 µl	
	50 µl	

Table 5. PCR profiles for primers: NS1/NS6, ITS5/LR7, JS1/JS8, LROR/LR7, ITS4/ITS5 and ITS1/ITS4

Primers	Cycle number	Temperature (°C)	Time
JS1/JS8, LROR/LR7, ITS5/LR7	35	94 °C	2 minute
		95 °C	1 minute
		52 °C	1 minute
		72 °C	2.5 minutes
		72 °C	10 minutes

NS1/NS6	35	94 °C	5 minute
		95 °C	5 minute
		55 °C	1 minutes
		72 °C	1.5 minutes
		72 °C	5 minutes
ITS1/ITS4. ITS4/ITS5	35	94 °C	2 minute
		95 °C	5 minutes
		55 °C	1 minutes
		72 °C	2 minutes
		72 °C	10 minutes

4. PCR product purification

The PCR product was purified directly follow the manufacturer's instructions of NucleoSpin^{R} Extract (MACHEREY-NAGEL). Then checking for the quantity and quality in a 1% agarose gel electrophoresis was applied. Finally, the purified PCR product was used directly for DNA sequencing.

5. DNA Sequencing

PCR products were directly sequenced by Macrogen., Inc in Korea using primers NS1, NS3, NS4, NS5, NS6, JS1, JS8, LROR, LR5, LR7, ITS1, ITS4, ITS5, RPB2-5F2 and RPB2-7CR (White et al., 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al., 1999).

6. Phylogenetic analyses

Fungal list with various taxa were analyzed along with other sequences obtained from the GenBank Database, with a suitable outgroup taxa and aligned initially with the computer program Bioedit (Hall, 2006) and Clustal W (Thompson et al., 1997) with default parameter settings, and alignments were manually edited by inserting gaps for optimization using Se-Al (Rambaut, 2002). Phylogenetic analyses of SSU rDNA, LSU rDNA, ITS region and RPB2 gene were performed with maximum parsimony employing a heuristic search (1000 random replicates) in PAUP* v 4.0b10 (Swofford, 2002). Ambiguously aligned regions also were excluded from the phylogenetic analyses. Maximum parsimony trees were found using 1,000 heuristic searches and including parsimony-informative characters in stepwise

(random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Branch support for all parsimony analyses was estimated by performing 1,000 bootstrap replicates (Felsenstein, 1985) with a heuristic search of 10 random-addition replicates for each bootstrap replicate. Descriptive tree statistics tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for all trees generated under different optimality criteria.

RESULTS

1. Phylogenetic analyses of the SSU dataset

To determine the taxonomic position and investigate the monophyly of the genera *Ascotaiwania, Canalisporium* and *Savoryella* at the ordinal level, the type species of *Savoryella* (*S. lignicola*) and *Canalisporium* (*C. careben se*) were also included in the 18S rDNA dataset. Thirty–two taxa of *Ascotaiwania, Canalisporium* and *Savoryella* from the BIOTEC Culture Collection (BCC) were aligned along with representative taxa from Class Sordariomycetes with three main Subclasses: Hypocreomycetidae, Sordariomycetidae and Spathulosporomycetidae. In subclasse Hypocreomycetidae, various taxa from four orders, consisting of the Halosphaeriales, Microascales, Hypocreales, Melanosporales and Hypocreomycetidae *incertae sedis* (unnamed clade) were included in the analysis, whereas seven major orders from the Subclasse Sordariomycetidae (Diaporthales, Coniochaetales, Chaetosphaeriales, Calosphaeriales, Ophiostomatales, Sordariales and Boliniales) and two taxa of the ascomycetes *incertae sedis* (*Pseudohalonectria falcata* and *P. falcate*) were incorporated with this study. Members of the order *Xylariales* (*Daldinia concentrica* and *Xylaria hypoxylon*) were chosen as the outgroup taxa for this data.

Maximum parsimony resulted in 18 most parsimonious trees (MPTs) with tree length (TL) 2309 steps, Consistency indices (CI) and Retention indices (RI), Homoplasy indices, respectively. Initial analysis of this dataset with a tree length of 2309 (CI=0.472, RI=0.846, RC= 0.400, HI=0.528) shown in Figure 3. A total of 1189 characters, 532 are parsimony informative, 497 are constant characters, 160 are variable character (parsimony uninformative).

The genera *Savoryella, Canalisporium* and *Ascotaiwania* formed a well supported clade (ACS clade) and clearly distinct from the Halosphaeriales, Hypocreales, Melanosporales, Miciroascales (Hypocreomycetidae) and Sordariales (Sordariomycetidae).

The four *Canalisporium* species (*C. caribense, C. elegans, C. pallidum* and *C. pulchrum*) and five *Savoryella* species (*S. aquatica, S. lignicola, S. longispora, S. paucispora* and *S. verrucosa*) formed a monophyletic subclade with a well-supported bootstrapping (Figures 3-4).

The *Ascotaiwania-like* sp. nov (SS03615 or BCC20507) grouped with the *Canalisporium* species, but this relationship did not receive any support. However, *C. exiguum* formed a basal clade to the the *Savoryella* subclade, with low support (76%).

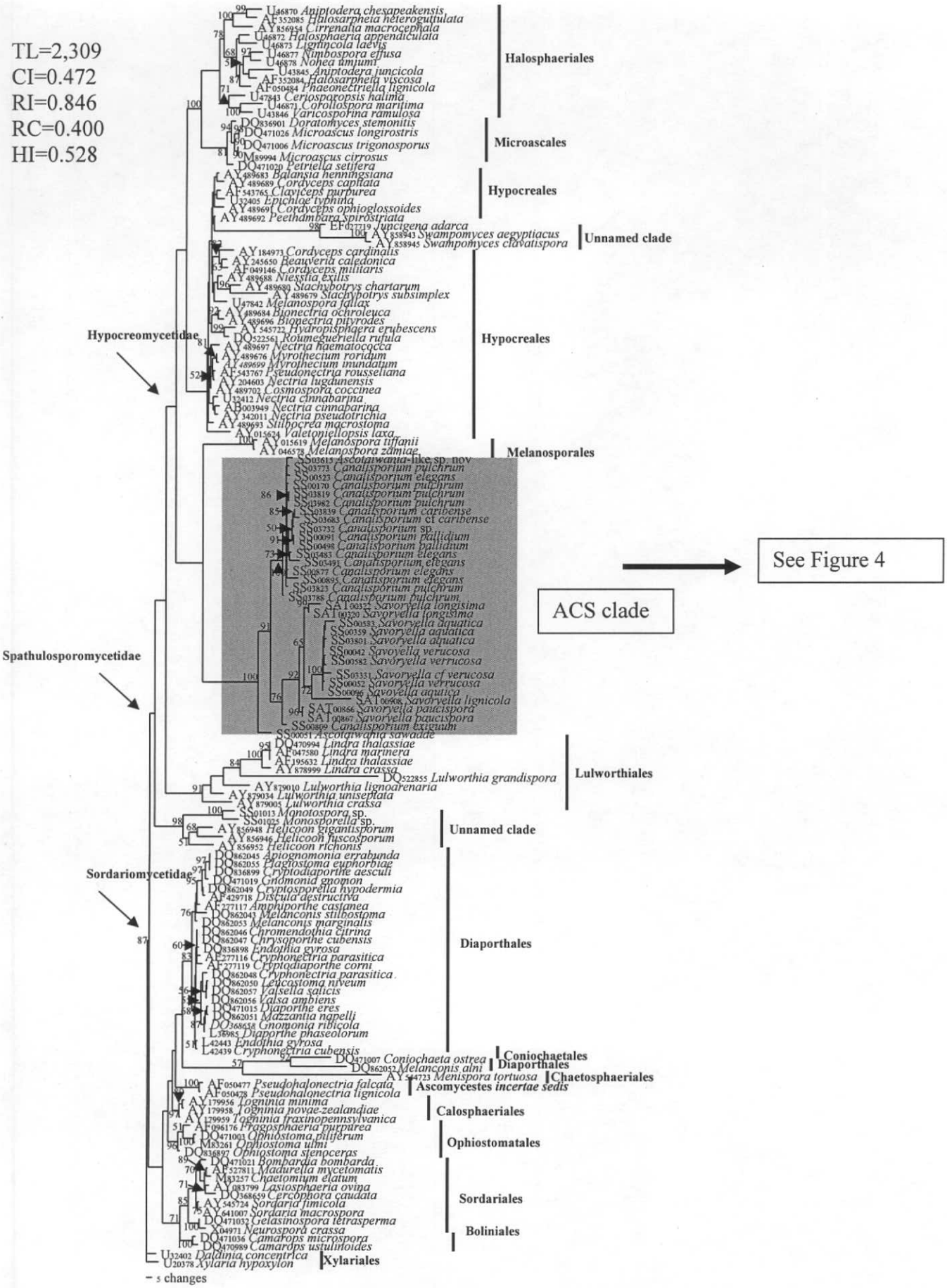

Figure 3 One of the 18 phylogenetic trees within the Sordariomycetes inferred from the maximum parsimony analyses of the SSU rDNA. Bootstrap values above 50% from 1,000 replications are designed above the corresponding nodes. The taxa known order names from NCBI are provided to the right of specie name.

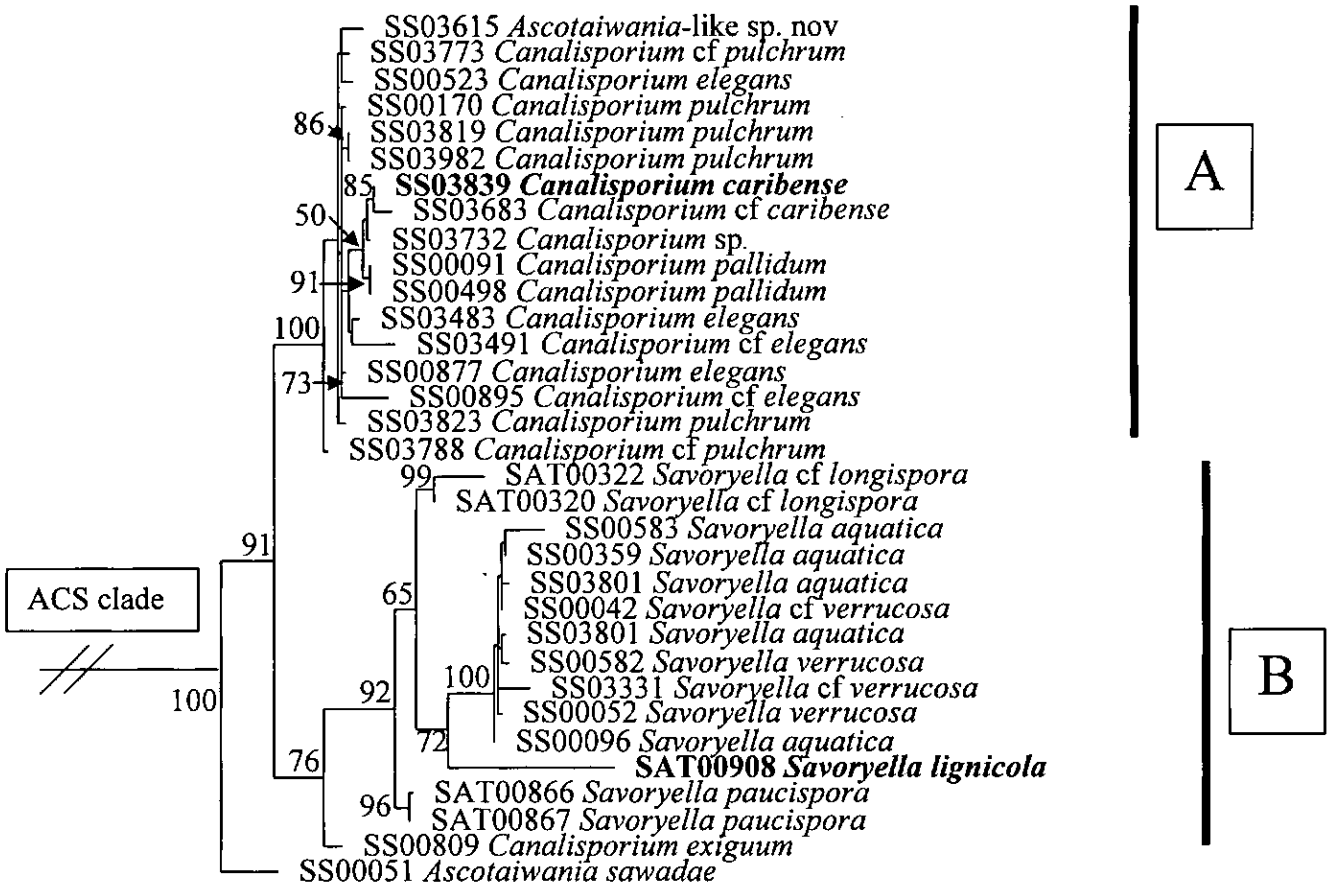

Figure 4 Phylogram derived from the MPs of SSU rDNA (excluding their related GenBank species). This dataset as described in figure 3. Numbers at the nodes are the bootstrap values with higher than 50% and the type species are denoted by the bold.

2. Phylogenetic analyses of the LSU dataset

The dataset of 28S rDNA sequences consisted of 33 taxa (*Savoryella*, *Canalisporium* and *Ascotaiwania*) from the BIOTEC Culture Collection (BCC). Further taxa (*Ascotaiwania hughesii, A. mitriformis, A. persoonii* and *Monotosporella setosa*) from the GenBank were also added to the analysis of the LSU dataset. A total of 1074 characters, 745 are parsimony informative, 119 are parsimony uninformative and 210 are constant characters. This dataset comprised representative taxa from the major order Corophorales, Hypocreomycetidae *Incertae sedis*, Halosphaeriales, Microascales, Hypocreales, Chaetosphaeriales, Boliniales, Sordariales, Coniochaetales, Calosphaeriales, Diaporthales, Ophiostomatales, Lulworthiales and confirmed that the ACS clade formed a well supported clade with affinities to the

Corophorales and Hypocreomycetidae *Incertae sedis* (Unnamed clade of *Torpedospora*, *Swampomyces* and *Juncigena*) as TBM clade. *Daldinia concentrica* (U47828) and *Xylaria hypoxylon* (U47841) from Order *Xylariales* were chosen as the outgroup taxa for this analysis based on 28S rDNA (LSU data). A total of 52 equally most parsimonious trees (TL=1995, CI=0.161, RC=0.095, RI =0.589 and HI=0.839) were obtained and compared for the best topology with the Kishino-Hasegawa test (Figure 5).

The first subclade A comprised *Savoryella* species with *S. lignicola*, the second subclade B included the genera *Ascotaiwania* and *Monotosporella* within the ACS clade, while the subclade C included *Canalisporium* species with the unnamed ascomycete fomed a monophyletic group (Figure 6).

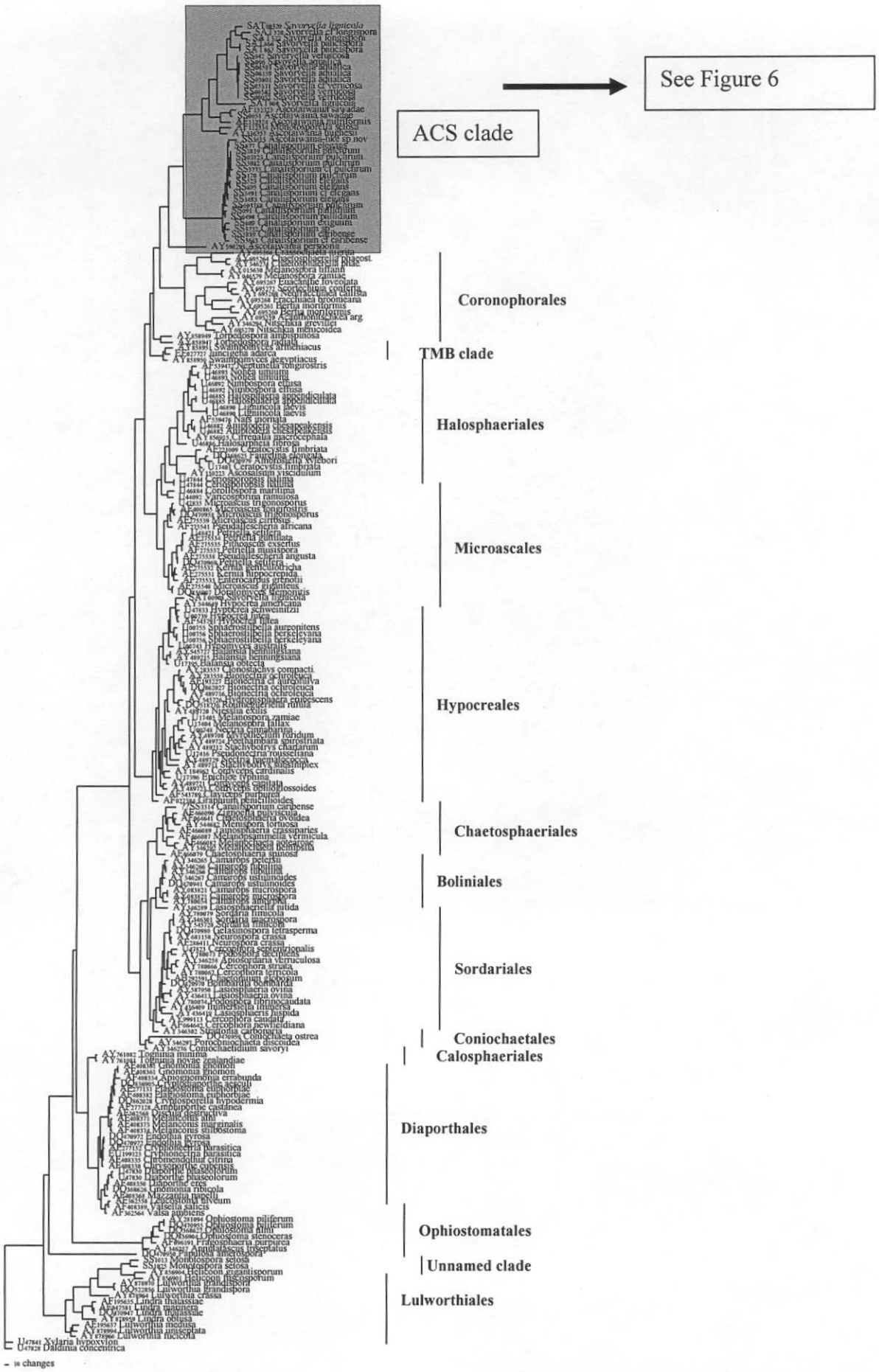

Figure 5 The best tree from the pasimonious tee depicting the relationship of species from the Sordariomycetes. Tree based on partial 28S rDNA sequences. All *Savoyoyella*, *Canalisporium* and *Ascotaiwania* species occur as a monophyletic group.

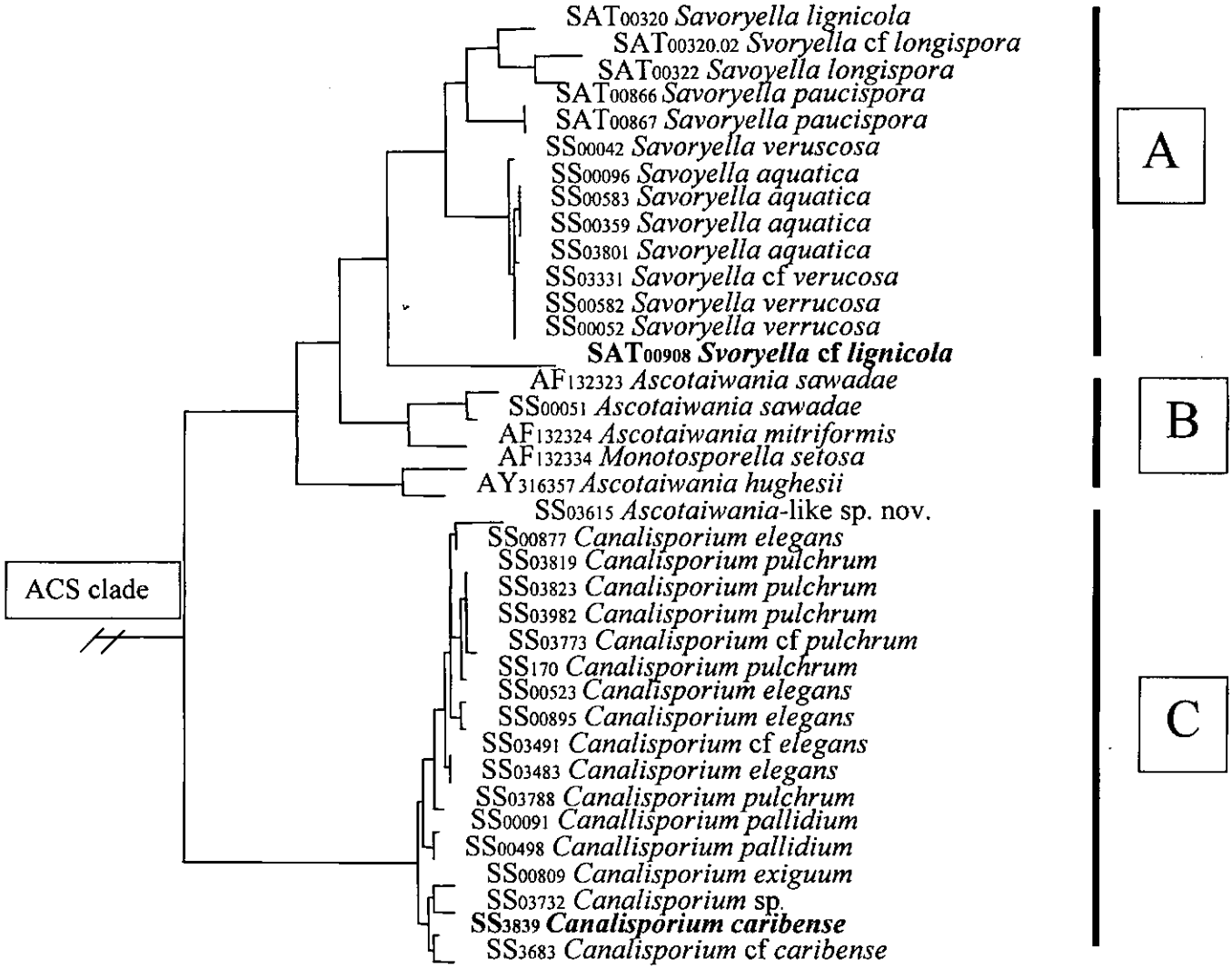

Figure 6 The tree derived from partial 28S rDNA sequences excluded all related species from GanBank. The type species sequenced in this study are shown in bold.

3. Phylogenetic analysis of the SSU+LSU dataset

The combined LSU/SSU dataset comprised 39 sequences with *Daldinia concentrica* and *Xylaria hypoxylon* as outgroup. One of the fifty-nine most parsimonious trees (MPT) was shown in Figure 7 with the most parsimonious tree, tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) as listed in Figure 7.

Third-teen sequences of *Savoryella* in this study clustered together including the type spices (*S. lignicola*) with high statistical support (100%). *Ascotaiwania* sequences separated into three groups: *A. sawadae* and *A. mitriformis* (AF132324), but the statistical support within this group is low (68%). *Ascotaiwania*-like sp. nov. (SS03615) clustered with various species in the *Canalisporium* clade with high bootstrap value (100%). *A. hughesii* grouped with *Monotosporella setosa* (AF132334). All *Canalisporium* species with seven-teen sequences formed a monophyletic clade with good support. Finally, three species of *Helicoon* formed a basal clade.

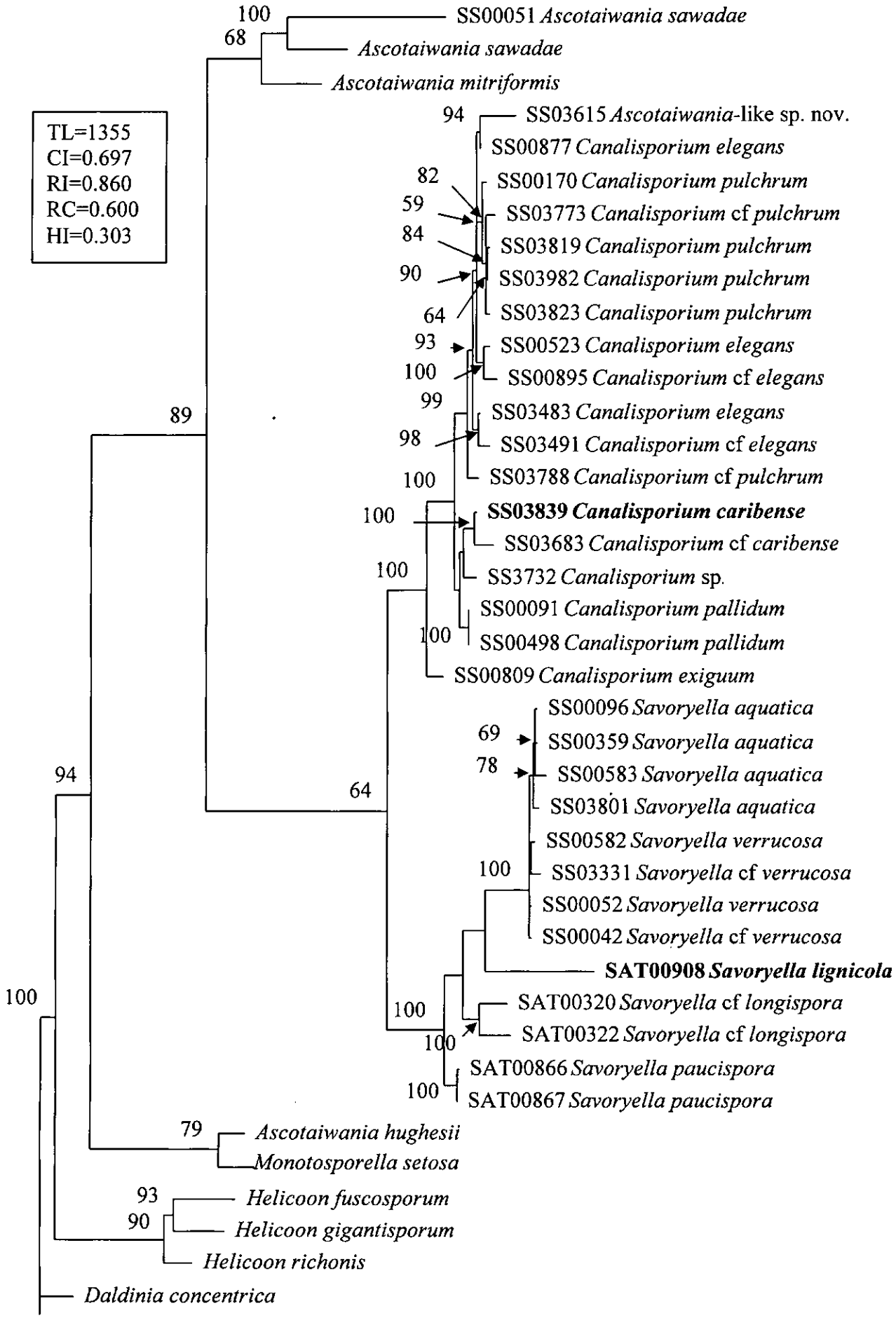

Figure 7 One of 59 most parsimony trees from the MP analysis based on combined SSU rDNA +LSU rDNA sequences. The tree is rooted with *D. concentrica* and *X. hypoxylon.* Values at each node are parsimony boostrap (above 50%). Related anamorphic taxa are denoted by GenBank accession numbers.

4. Phylogenetic analyses of the RPB2 dataset

In order to further establish the familial-ordinal status the genera *Savoryella* and *Canalisporium*, an alternative RPB2 gene, was selected. In the RPB2 dataset, 22 strains were sequenced, including *Savoryella* (5 sequences), *Canalisporium* (15 sequences) and *Ascotaiwania* (2 sequences). Maximum parsimony analysis yielded 12 maximum parsimonious trees with a length of 11293, Consistency Index of 0.156, Homoplasy Index of 0.844, Retention Index of 0.572 and Rescaled Consistency Index of 0.089. One of the 12 most parsimonious trees is shown in Figure 8 with representative taxa from 12 major orders of the Sordariomycetes comprising the unnamed clade (Hypocreomycetidae *Incertae sedis*), Halosphaeriales, Microascales, Hypocreales, Sordariales, Boliniales, Chaetosphaeriales, Coniochaetales, Ophiostomatales, Diaporthales, Lulworthiales and two *Xylaria* species (Xylariales) as the outgroup.

Phylogenetic analysis revealed that *Savoryella* (5 sequences), *Canalisporium* (15 sequences) and *Ascotaiwania* (2 sequences) form a monophyletic group with a 99% bootstrap support and separate from the Hypocreales, Halosphaeriales and Sordariales (Figure 8). Within the ACS clade, three subclades are discernable (Figure 9). *Savoryella* species (subclade A) grouped with high statistical support, *Canalisporium* species (subclade B) grouped with high boostrap support and included the *Ascotaiwania*-like sp. nov. (SS03615) with *A. sawadae* (SS00051) (Figure 9) grouped with unidentified *C. caribense* strain in subclade C. This data is in good agreement with 18S rDNA and 28S rDNA for assessing the taxonomic position in the Hypocreomycetidae *incertae sedis*, Sordariomycetes, although some of the clades/subclade obtained different taxa from GenBank.

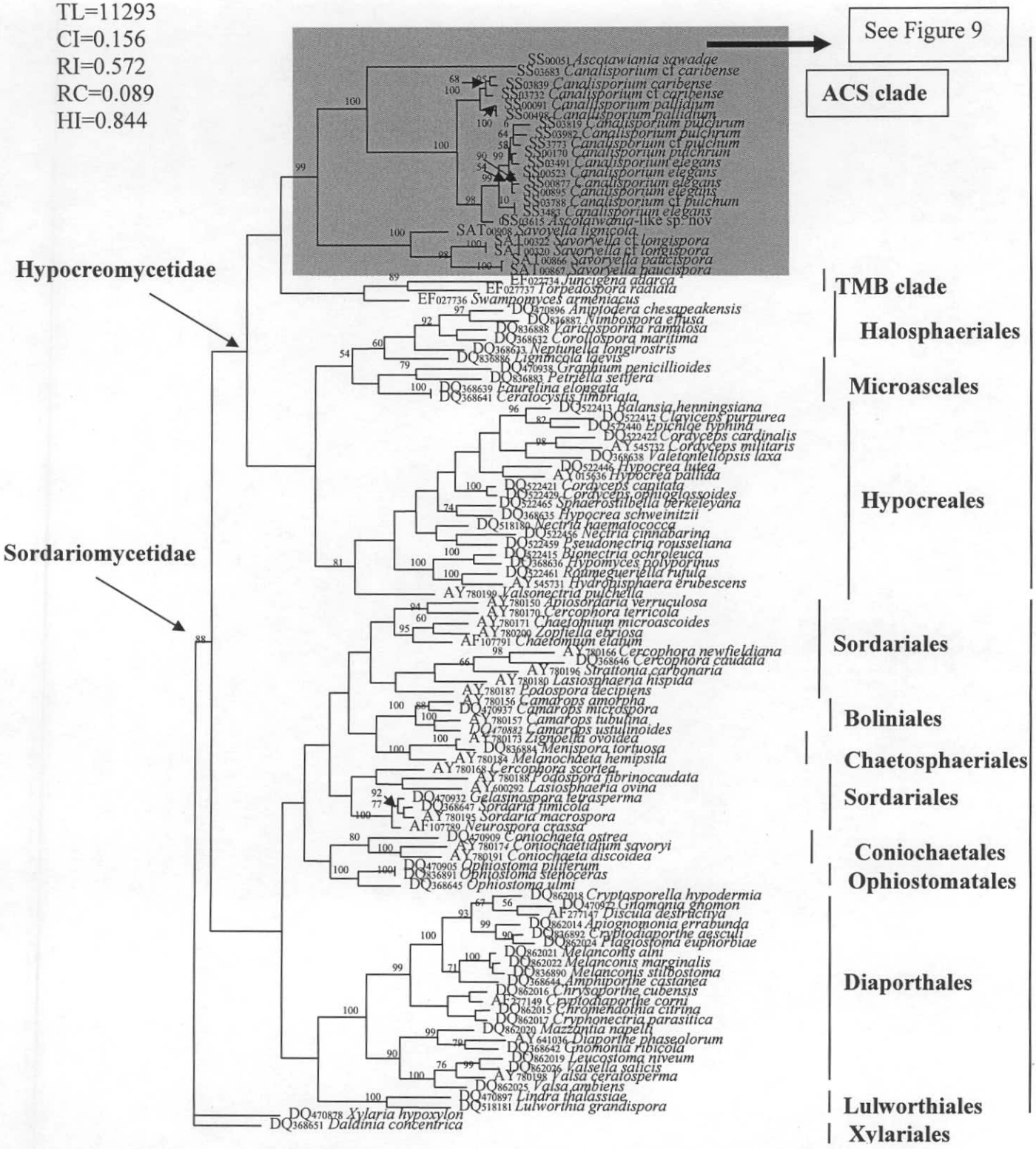

Figure 8 Phylogram using RPB2 gene showing phylogenetic relationship between related species from the GenBank and species of *Savoryella*, *Canalisporium* and *Ascotaiwania*. The numbers on the nodes represent the percentage boostrap support based on pasimony analysis The RPB2 sequences are indicated by their GenBank accession in the figure. The phylogeny was rooted with the order Xylariales as outgroups.

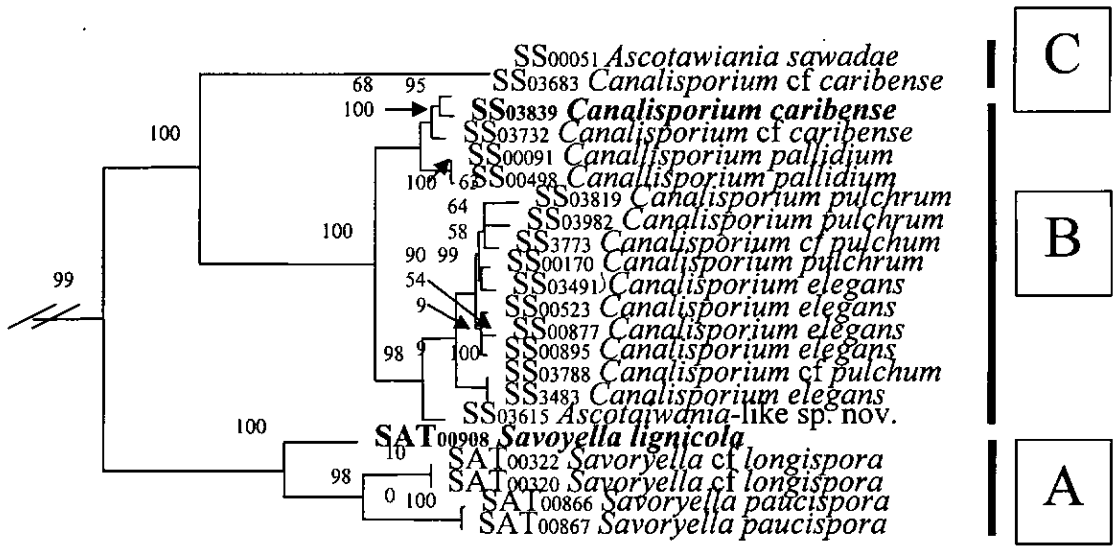

Figure 9 Phylogenetic relationships between *Savoryella* spp., *Canallisporium* spp. and two taxa of *Ascotaiwania* derived from MPT of the RPB2 gene (excluding their related GenBank species). This dataset as described in figure 8. The resulting bootstraps greater than 50% are shown above branches. The type species of genera (*Canalisporium* and *Savoryella*) are printed in bold.

5. Phylogenetic analyses of the individual LSU gene dataset

From the preliminary analysis of the data for *Savoryella, Canalisporium* and *Ascotaiwania*, agree with (18S rDNA+GenBank dataset, the 28S rDNA+GenBank dataset and the RPB2+GenBank dataset). *Savoryella* and *Canalisporium* species in this study form a monophyletic clade within the Subclass Hypocreomycetidae, the Class Sordariomycetes. This 28S rDNA dataset is to investigate the phylogenetic relationship of the genera *Savoryella* (five sequences from Thai marine isolates and eight sequences from Thai aquatic isolates), *Ascotaiwania*-like sp. nov. (SS03615), *Canalisporium* (17 sequences) and *A. sawadae* (SS00051).

Five sequences from the GanBank (*Monotosporella setosa* AF132334, *A. hughesii* AY316357, *A. sawadae* AF132323, *A. mitriformis* AF132324 and *A. persoonii* AY590295) were also added to this analysis with two *Xylaria* species as the outgroup. The number of most parsimonious trees (MPT), tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and

homoplasy index (HI) are listed in Figure 10. Total of a 1241 characters, 289 are parsimony informative, 812 are constant characters.

The Kishino–Hasegawa (K–H) test was used for estimation of the best tree topology MP analysis shown in Figure 10. The tree originated by unweighted parsimony analysis yields the best KH-likelihood scores shown in the Figure 10. All topologies are similar to the phylogeny generated from the ITS dataset (data not shown). According to our analyses, our sequences based on the LSU rDNA data are divided into at least three major clades. Representative clades with bootstrap support values (BS) above 50% were designated as follows:

Clade A (*Savoryella* clade): *S. lignicola* (SAT00908), *S. longispora* (SAT00320, (SAT00322), *S. paucispora* (SAT00866, SAT00867), *S. veruscosa* (SS00042), *S. aquatica* (SS00096, SS00583, SS00359, SS03801), *Savoryella* cf *verucosa* (SS03331) and *S. verrucosa* (SS00582, SS00052). The clade is composed of two distinct groups of species (A1: marine-derived *Savoryella* species and A2: freshwater-derived *Savoryella* species); both are characterized by their habitat origin. Most of the internal nodes of each clade have moderate to high bootstrap support (51-100%) indicating that within each group, they are closely related. Within this clade, the first group of the marine species (A1) were represented by *S. lignicola* (SAT00908), *S. longispora* (SAT00320, SAT00322), *S. paucispora* (SAT00866, SAT00867), while the second group (A2) comprises two species of *Savoryella* (*S. aquatica* and *S. verrucosa*) originate in a freshwater environment collected from submerged wood.

Clade B *Canalisporium* consist of *Ascotaiwania*-like sp. nov. (SS03615), *C. elegans* (SS00877), *C. pulchrum* (SS03819, SS03823, SS03982, SS00170, SS03788), *Canalisporium* cf *pulchrum* (SS03773), *C. elegans* (SS00523, SS00895, SS03483), *Canalisporium* cf *elegans* (SS03491), *Canalisporium* sp. (SS03732), *C. exiguum* (SS00809), *C. caribense* (SS03839), *Canalisporium* cf *caribense* (SS03683) and *C. palladium* (SS00091, SS00498).

The *Canalisporium* species are considered monophyletic, but again divide into 2 groups: B1 comprises most of the speices while *C. palladium* forms a sister group with high support. *Ascotaiwania* species do not form a monophyletic clade. *A. hughesii* and its anamorph formed a sister group to the *Savoryella*/*Canalisporium*

clades, while *A. sawadae* and *A. mitriformis* formed a separated clade to the *Savoryella/Canalisporium* clade.

Clade C "*Ascotaiwania*" spp., comprise *A. sawadae* (SS00051), *M. setosa* (AF132334), *A. hughesii* (AY316357), *A. sawadae* (AF132323), *A. mitriformis* (AF132324) and *A. persoonii* (AY590295), form a sister group with Clade A and B. Most taxa are sequences derived from the GenBank. Within this Clade, *A. persoonii* (AY590295) is basal to all other taxa but without any support (subclade C3). The grouping of *A. sawadae* (SS00051) and *A. sawadae* (AF132323) is 100%, while *A. mitriformis* (AF132324) forms as a basal sister taxon (bootstrap values= 84%) in subclade C2, with other taxa *M. setosa* (AF132334) and *A. hughesii* (AY316357) in the subclade C1 with a weak support (bootstrap values= 53%). Within subclade C1, *M. setosa* (AF132334) and *A. hughesii* (AY316357) are closely related with high bootstrap support.

In this study, *Savoryella* species and *Canalisporium* species form a monophyletic groups (within the subclass Hypocreomycetidae, the Class Sordariomycetes), with *Ascotaiwania* spp. as a sister clade. The exception is *Ascotaiwania*-like sp. nov (SS03615).

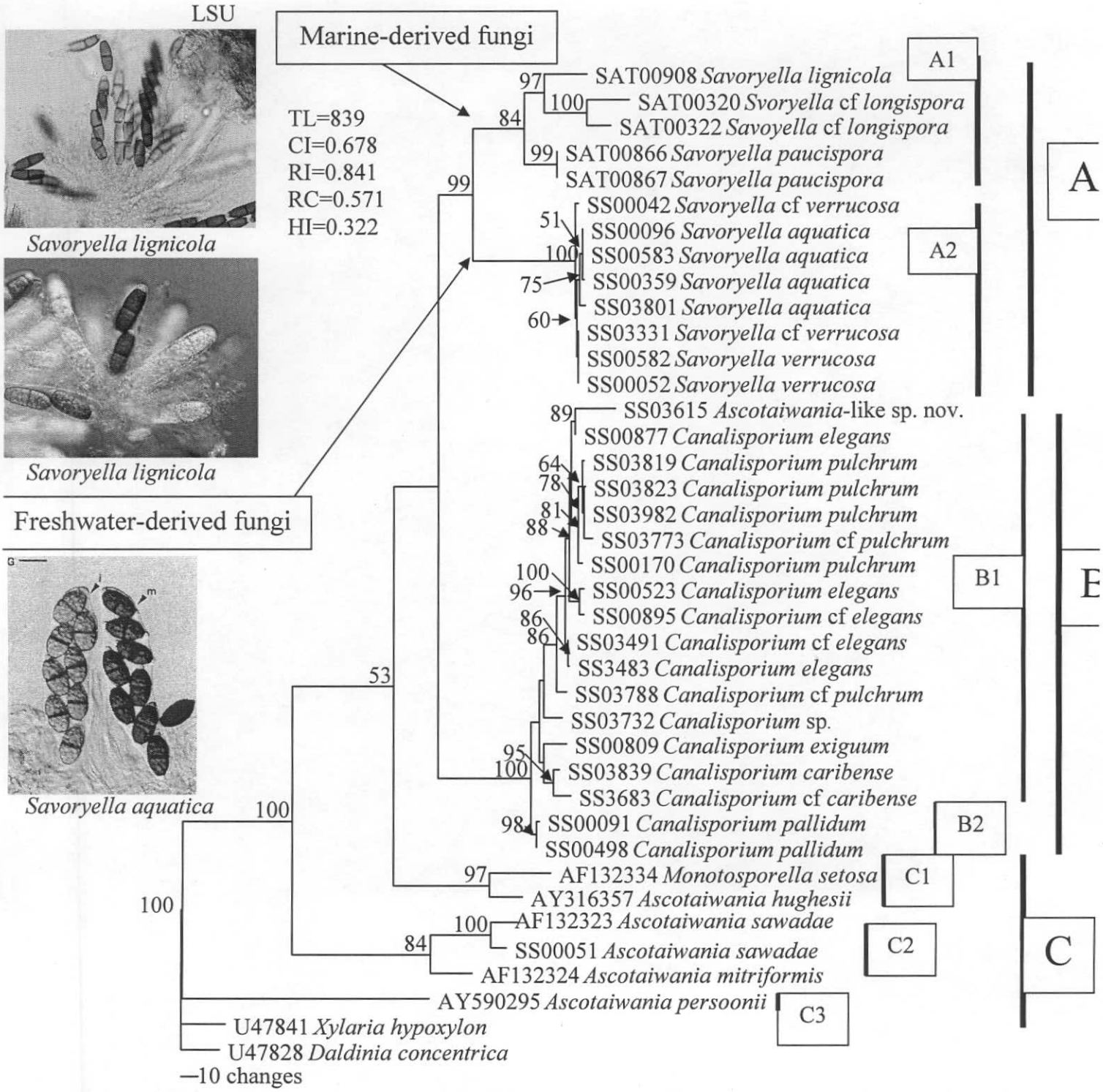

Figure 10 The phylogram of the best tree inferred from the maximum parsimony analyses of 28 rDNA data. Bootstrap value greater than 50% is given above the corresponding nodes. The out group taxa are *Xylaria hypoxylon* and *Daldinia concentrica*.

6. Phylogenetic analysis of the individual ITS sequence dataset

The ribosomal (ITS1, 5.8S, ITS2) sequence dataset was analyzed by parsimony analysis. The resulting dataset comprised 35 sequences; with *Xylaria hypoxylon* (FJ205468) and *Daldinia concentrica* as the outgroup taxa. Initial analysis of this dataset yielded 46 trees with a tree length (TL) of 1693 (CI= 0.615, RI= 0.808, RC=0.497, HI=0.385) shown in Figure 11. A total of 758 characters, 491 are parsimony informative and 196 are constant characters.

In the analysis of the ITS sequence (the genera *Canalisporium* and *Savoryella*) showed a common node with the bootstrap (67%).

Fifteen *Canalisporium* formed a well-supported monophyletic clade strongly support by 100% bootstrap with *Savoyella* species grouped as a siter clade. The two *A. sawadae* strains were monophylytic with 85% bootstrap support. Twelve *Savoryella* species constitute a well-supported monophyletic clade with a bootstrap value of 97% and appeared to be phylogenetically distinct from other genera such as *Canalisporium*, *Monotosporella*, *Ascotaiwania* and *Helicoon* (Figure 11). Within the *Savoryella* clade, most of the internal subclades did not receive reliable branch support. The Thai marine strains *Savoryella* cf *longispora* (SAT00320) and *S. paucispora* (SAT00866, SAT00866) grouped together, but with weak statistical confidence. However, the position of *S. lignicola* (SAT00908), the type species and a marine isolate, did not cluster with other *Savoryella* derived from marine habitats. Instead, it was basal to other Thai freshwater *Savoryella* species. In the Thai freshwater *Savoryella* subclade, four isolates of *S. aquatica* group consistently with 85% bootstrap support, while *S. verrucosa* clusters separately in this subclade with 86% bootstrap support. *Monotosporella* strains and two *Helicoon* strains did not group with the *Ascotaiwania* strains.

The congruence of ITS rDNA and LSU rDNA datasets derived phylogenies was tested by analyzing the respective dataset independently with both Bayesian (data not shown) and parsimony. Separated parsimony phylogenetic analyses of the ITS region dataset and partial LSU dataset resulted in similar topologies, both data providing better resolution of deeper nodes.

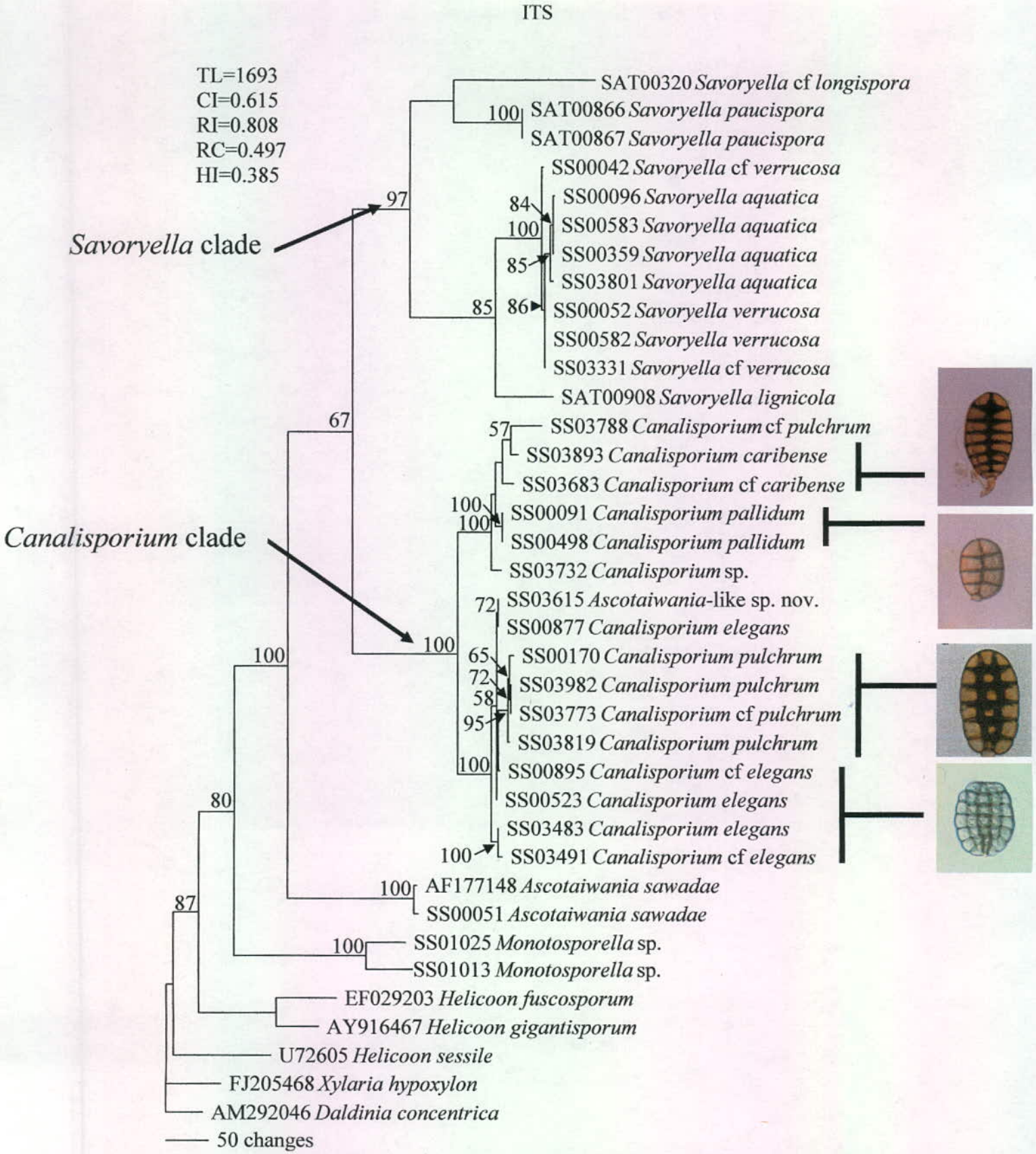

Figure 11 The phylogram from MP analysis of *Savoryella*, *Canalisporium*, *Ascotaiwania* and their anamorph of *Ascotaiwania* (*Monotosporell setosa* and *Helicoon* spp.) rooted with *Xylaria* and *Daldinia*. Bootstrap values greater than 50% are indicated along nodes.

7. Phylogetic analyses of the individual RPB2 and RPB2+ITS dataset

Twenty-two *Ascotaiwania, Canalisporium* and *Savoryella* sequences were included initially with *Daldinia concentrica* (DQ470878) and *Xylaria hypoxylon* (DQ368651) as the outgroup. In five parsimony analyses, the tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) listed in Figure 12. Like the results from ITS flanking 5.8S dataset (Figure 11), when gaps were totally excluded, grouping topologies of *Canalisporium* and *Savoryella* clades were alike, but this analysis is limited because of the sequences available for study. *Savoryella* clade A (four *Savoryella* strains) and *Canalisporium* clade B (various *Canalisporium* species) form a single clade with *A. sawadae* (SS00051) as a basal taxon to *Canalisporium* species.

Maximum parsimony analysis of the combined dataset (ITS+RPB2 dataset) groups all *Canalisporium/Savoryella/Ascotaiwania* taxa into three clades (Figure 13) with bootstrap values showed above the branch at each node. The topology of the three clades are the same as for individual ITS-5.8S rDNA dataset, individual 18S rDNA dataset, individual 28S rDNA dataset and the combined dataset showed that *Ascotaiwania* taxa formed a basal clade to both *Canalisporium* and *Savoryella* species. But the position of the *Ascotaiwania* clade was ambiguous and was basal to the *Canalisporium* species but with weak support.

Canalisporium strains formed a monophyletic group with *A. sawadae* as a sitster clade to the *Canalisporium* group, while in the *Savoryela* clade freshwater strains grouped together with the marine strains in an adjacent group.

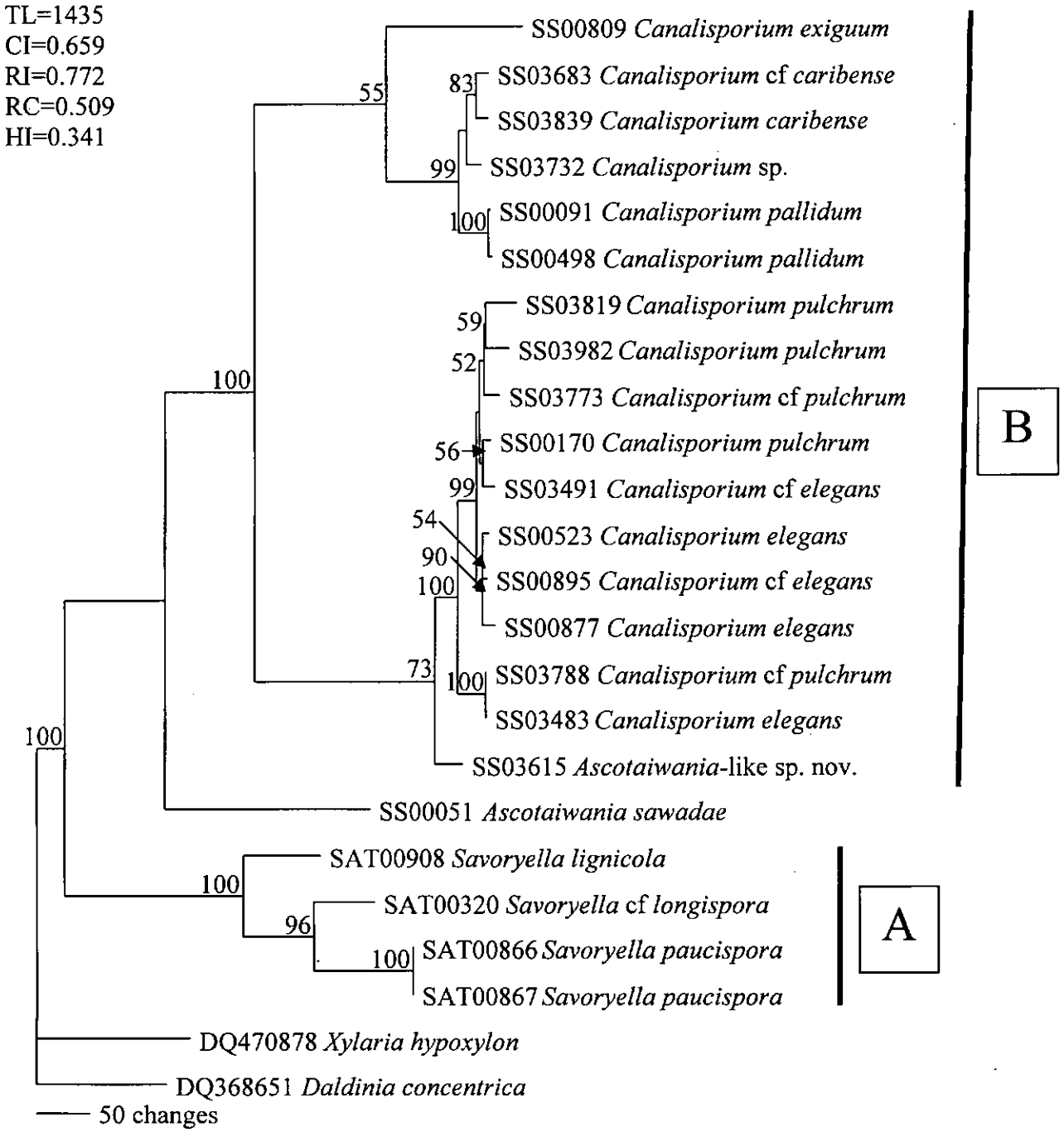

Figure 12 The phylogram obtained from partial RPB2 gene analysis. Bootstrap value higher than 50% from maximum parsimony analysis are given above the branches.

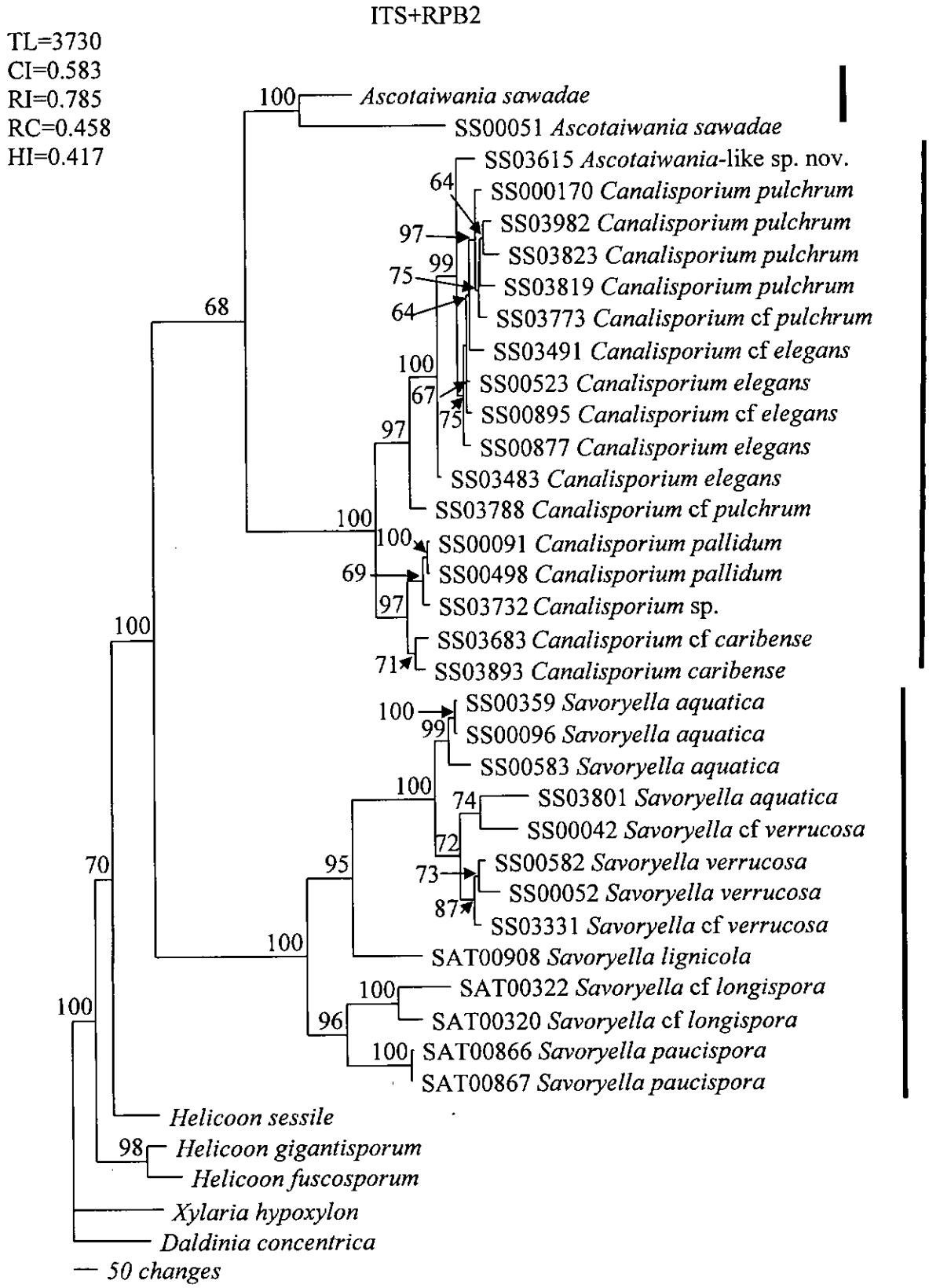

Figure 13 One of 3 the most parsimonious trees generated from combined data of the ITS+RPB2 sequences. Numbers at the nodes are the bootstrap value.

8. Phylogenetic analysis of the ITS+LSU dataset

The complete alignment of ITS1-2, 5.8S and partial LSU nu-rDNA sequences in this dataset yielded 208 most parsimonious trees, with TL=2499, CI=0.635, RC=0.519 and HI=0.365. The KH test model of 208 trees indicated that the three from unweighted parsimony analysis with an estimated shape parameter yielded the best phylogenetic hypothesis for this study, the best phylograms of which is shown in Figure 14. The KH test showed that these trees were not significantly different. *Canalisporium* and *Savoryella* strains formed adjacent clades with 54 % bootstrap support.

Ascotaiwania strains are not mononphyletic with *A. sawadae* and *A. mitriformis* as a sister group to the *Canalisporium/Savoryella* clades, but with no support. *Ascotaiwania hughesii* and *A. persoonii* were distantly placed and formed separate group to the *Canalisporium/Savoryella* clades

One of 208 MP: ITS and LSU

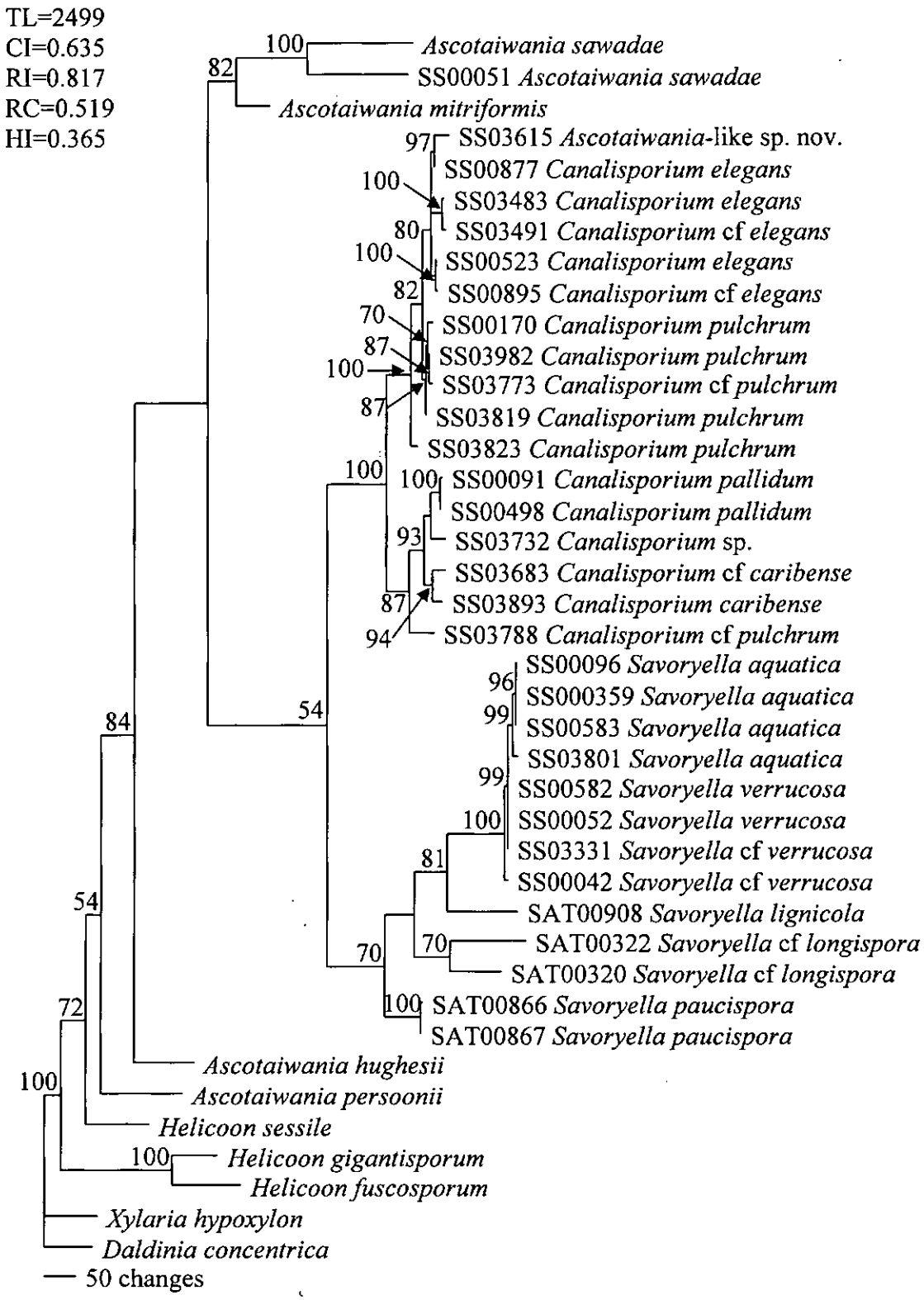

Figure 14 Phylogram of one of the 208 equally most parsimonious trees obtained from the parsimony analysis based on combined ITS rDNA and 28S rDNA sequences. The tree rooted with *Xylaria hypoxylon* and *Daldinia concentrica* (the Xylariales).

9. Phylogenetic analysis of the SSU+LSU+RPB2 dataset

The combined SSU+LSU+RPB2 dataset (based on maximum parsimony analysis) was computed with the SSU+GenBank, individual LSU, the ITS, combined SSU+LSU, RPB2, the combined ITS+RPB2 and the ITS+LSU rDNA datasets, in order to compare the tree topology.

The sequence data in this analysis as a combined dataset consisted of 3369 characters, 1053 are parsimony informative, 390 were variable (parsimony uninformative) and 1926 were constant. Initial analysis of this dataset yielded 8 trees with a tree length of 3528 (CI= 0.613, RI= 0.803, RC= 0.492, HI=0.387 shown in Figure 15.

Ascotaiwania strains are polyphyletic with *A. sawadae* and *A. mitriformis* as a sister group to the *Canalisporium/Savoryella* clades, but with weak support. *Ascotaiwania hughesii* and *A. persoonii* formed separate clades to the *Canalisporium/Savoryella* clades. *Canalisporium* strains formed a monophyletic group with *A. sawadae* as a sitster clade.. Five *Savoryella* species (*S. aquatica*, *S. lignicola*, *S. longispora*, *S. paucispora* and *S. verrucosa*) formed a monophyletic subclade with high bootstrap support.

TL=3528
CI=0.613
RI=0.803
RC=0.492
HI=0.387

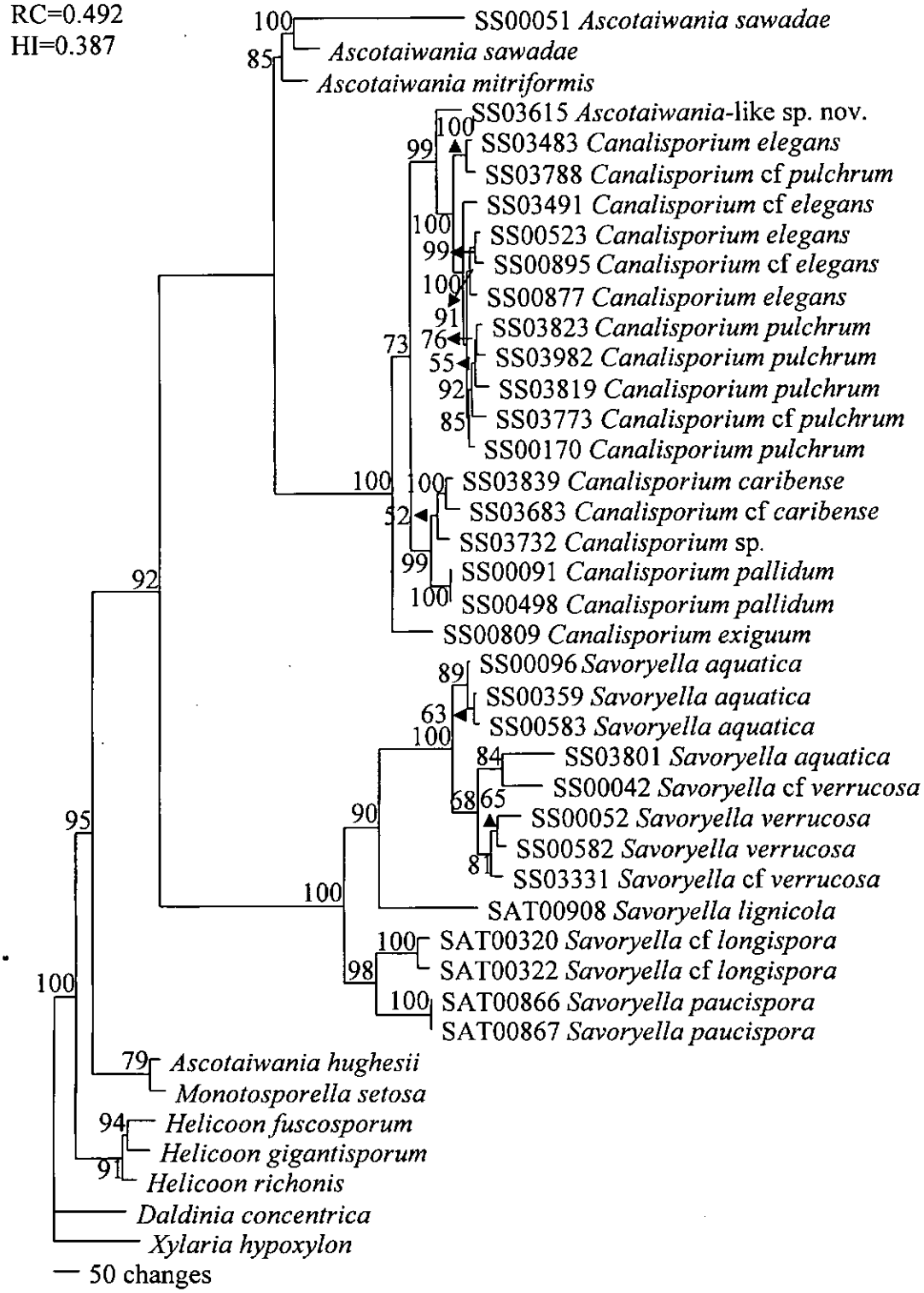

Figure 15 Combined ribosomal and protein phylogeny (SSU rDNA, LSU rDNA, RPB2). The placement of the *Canalisporium/Ascotaiwania/Savoryella* together with their anamorphic taxa. The tree is the most parsimonious trees. The tree rooted with *Xylaria hypoxylon* and *Daldinia concentrica* from the Order xylariales Bootstrap values higher than 50% from maximum parsimony analysis are given above nodes.

DISCUSSION

1. A new lineage of the ACS clade

Hibbett et al (2007) accepted three subclasses in the Sordariomycetes: Hypocreomycetidae (with the orders Coronophorales, Hypocreales, Melanosporales, Microascales); Sordariomycetidae (with the orders Boliniales, Chaetosphaeriales, Coniochaetales, Diaporthales, Ophiostomatales, Sordariales) and the Xylariomycetidae (with the order Xylariales), while the orders Lulworthiales, Meliolales, Phyllachorales and Trichosphaeriales are represented as Sordariomycetes *incertaesedis*.

The genera *Ascotaiwania*, *Canalisporium* and *Savoyella* studied here formed a clade (here after referred to as ACS) within the Hypocreomycetidae with the Coronophorales and the TBM clade as sister clades. They form a distinct clade to the order Halosphaeriales, Microacales and Hypocreales, whereas genera grouping in the TBM clade are morphologically diversed to those in the ACS clade. The ACS clades have a numbers of shared features: ascomata generally swan-like shaped rarely with a central neck, unitunicate asci, that are persistent, clavate to cylindrical, short pedunculate with without paraphyses, generally with an apical pore, ascospores, asci cells, cell hyphae-like, central cells brown. Most ascospore appendages are lacking except for the marine species of *S. appendiculata.*

All are saprobes; most are aquatic and well growing on decayed wood as lignocellulose materials (Sivichai et al., 2002, 2003). However, few are active degrades of lignicelluose (Jones & Eaton 1969). The ACS clade represents yet another new lineage of the Hypocreomycetidae. It is interesting that both the TMB and ACS clades occur in aquatic habitats, transitional from tesrestrial to freshwater to brackish and fully saline habitats.

Although the ACS clade represents a new lineage of ascomycetes, it is premature to elect a new order to accommodate this group of taxa.

No anamophs have been reported for *Savoyella*, while several and dematiaceous hyphomycetes have been reported to the genus *Ascotaiwania*: *Monotosporella* sp. (*A. sawadae*; Sivichai et al., 1998), *M. setosa* (*A. sawadae*; Ranghoo et al, 1999) and *Helicoon* (*A. hughesii*; Fallah et al., 1999; Tsui and Berbee,

2006). In our analyses, *Ascotaiwania* is not monophyletic, although they form a distinct group (Ranghoo et al, 1999; Cambell and shearer, 2004).

2. Order placement of *Savoryella* and *Canalisporium* species

Our current study expands the Vijaykrishna et al (2006) dataset with additional sequences within a broader taxomomic and phylogenetic samplings of Sordariomycetes. Therefore, the tree from 18S rDNA will be discussed based on the ordinal position.

The phylogenetic position of *Savoyella* and *Canalisporium* generated from MP methods were similar under different genes (phylogenetic topology with the LSU dataset, the RPB2 dataset and the combined gene dataset) and the branching patterns with respect to the placement of ingroup taxa were similar to those obtained from SSU nu-rDNA phylogeny (Zhang et al, 2006; Schoch et al, 2007; Tang et al, 2007) although some of the clades/subclades obtained different taxa from GenBank.

Our results cleary show that *Savoyella* having morphological same as *Ascotaiwania* (Sordariales *Incertae sedis*, Sordariomycetidae) does not phylogenetic affinity with the Hypocreales within the Hypocreomycetidae (Vijaykrishna, 2005) and Cai et al, 2006). This suggestion should be assigned to other orders and with *Ascotaiwania*, in a sister clade. It is best referred to the Hypocreomycetidae *incertae sedis*, Sordariomycetes. These findings suggest a new lineage of aquatic ascomycetes that have invaded both the marine and freshwater habitates. Although these genera are related, tree topologies between the differenet datasets vary as they contain differenet taxa. They form a distinct group similar to the unclassified group of marine ascomycetes comprising *Swampomyces, Torpedospora* and *Juncigera* (Sakayaroj et al 2005; Schoch et al 2007).

3. The monophyly of the genera *Savoryella/Canalisporium*

The genus *Savoryella* is one of the most commonly reported unitunicate ascomycete genus from submerged wood in rivers or streams (Sivichai et al., 2002, 2003). All analyses, the monophyly of *Savoryella/Canalisporium* are supported, but the phylogenetic assignment of those genera is unresolved as it has been referred to a number of orders and families in the Sordariomycetes, Sordariomycetidae.

Morphologically, the genus *Savoryella* resembles *Ascotaiwania* Sivan. & H. S. Chang and shows to share few traits in common with genera *Ascotaiwania* with its versicolourous ascospores but differs in having cylindrical asci with a relatively massive, non-amyloid apical ring, ascospores that are 4-8-septate (Chang et al 1998). No anamorph are known for described species of *Savoyella*. In particular we found that *Savoyella* and *Canalisporium* are related phylogenetically with *Ascotawania* as a basal clade. In our results, *Ascotaiwnaia* is polyphyletic with the differnet species grouping with differenet anamorphs (*Monotosporella, Helicoon* and *Canalisporium*) and distantly formed with *Savoryella/Canalisporium* species.

The genus *Savoryella* clusters with *Canalisporium* species (18S rDNA phylogenies); however placement of the *Ascotawania* and our new taxon *Ascotaiwania*-like sp. nov. (SS03615) formed basal to other members of both genera.This new Ascomycete, showing similarities to *Ascotaiwania*, groups in all anlayses with *Canalisporium* speices and may be a new genus. Howerver, this topology of a new genus showed not grouped with *Ascotaiwania* species, comparing with a with the tree result based on 28S rDNA from (Ranghoo et al, 1999; Cambell and Shearer, 2004). This relationship, together with closely related genera, lacked statistical support and remains unresolved. Due to limited availability of sequences from databases, the phylogenetic relationship among *Savoryella/Canalisporium* and *Ascotawania* species cannot be ascertained, as well as lacked of type speices of *Ascotawania* for comparing in this study.

In ITS data, the majority of the internal nodes are supported by bootstrap analysis. Both selected freshwater and marine *Savoryella* species formed a monophyletic and separately with each other based on their origin of habitats. In the parsimony, most taxa of *Savoyella*, including *S. lignicola* as a type strain of *Savoyella* including *S. aquatica*, *S. lignicola*, *S. longispora*, *S. paucispora* and *S. verrucosa* were sorted into a large cluster, showed monophyletic clade. *S. aquatica* and *S. verrucosa* formed a strongly supported branch with each species supported by statistics and concordance. The *S. lignicola* (as a types specie of genus), *S. longispora* and *S. paucispora* linearege derived form marine origin is highly well supported as sibling taxa of *Savoyella aquatica* and *S. verrucosa* collected from freshwater stream.

This analysis is with the agreement topology with the independent analyzes based on the position of the *Savoyella* clade and *Canalisporium* clade showing not

polyphyletic genera. In contrast to the independent RPB2 dataset and their combined RPB2 dataset, there had some conflict of the topology resulting of *Ascotaiwania* spp. in the MP showing tree of ambiguous topology.

In the phylogenetic data, ITS analysis revealed that the *Savoryella* constitute a well supported and appeared to be phylogenetically distinct from other genera *Canallisporium*, *Ascotaiwania*, *Monotosporella* and *Helicoon* strains, which were spited across subclade of this study. The position of *Savoryella aquatica* and *Savoryella verrucosa* were grouped together with a well-supported boostrap and baysian and related to their habitat origin originated from freshwater environment

In our study the molecular characters (ITS ribosomal DNA sequences data), it is indicated that ITS data confidently be used to distinguish *Canalisporium* species. Additional analysis of ITS sequences suggested that ITS region with a greater number of species having a broader representation of the morphological variation present in the genus. Likewise, inclusions of additional species that are more restricted in their differing ecological habitats. However, some associations were observed among species groupings on the ITS tree need more type strains for inferring phylogenetic relationships among members of the *Canalisporium* and related genera that can have a strong effect on phylogenetic inference.

FUTURE WORK

Further studies on phylogenetic relationships among those species need to be carried out in order to establish accurately species limits. There were still many limitations because there have not many strains this congeneric genus to compare. Additionally, we should use other multi-gene analyses for identifying the taxonomic position to support our data.

Related species of *Savoryella* (teleomorph ascomycete) will be sequenced and analyzed via multiple gene methods, such as RPB1, EF1-α and β-tubulin for identifying of these genera and related species. We will combine sequence data with morphological traits and ecological characters to address the evolutionary question.

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PART III

POSTER BRT AND ABSTRACT OUTPUT

PART III

POSTER BRT AND ABSTRACT OUTPUT

Relationship of the genus *Savoryella* (teleomorph ascomycete) and its anamorph *Canalisporium* as inferred by multiple gene phylogenies

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The taxonomic placement of selected freshwater *Savoryella* species and some marine *Savoryella* species as well as putative *Canalisporium* species that originated from submerged woods in aquatic habitats have not been classified into any family or order with certainty. Results based on individual molecular data analyses of the partial small sequence (SSU data), indicate that *Savoryella* form a monophyletic clade and group within the subclass Hypocreomycetidae, Sordariomycetes. The genus *Savoryella* shows no affinities with the Hypocreales despite earlier assignment to that order. In addition, we can confirm using the large subunit rRNA gene (28S rDNA) the taxonomic position within Hypocreomycetidae, which is in good agreement with the 18S rDNA gene. Further analyses will be conducted including more strains of these taxa, and combining molecular analyses, such as ITS, RPB1, RPB2 and EF1-α, for determining the precise taxonomic placement of these genera.

RELATIONSHIP OF THE GENUS *SAVORYELLA* (TELEOMORPH ASCOMYCETE) AND THE ANAMORPH GENUS *CANALISPORIUM*, AS INFERRED BY SSU AND LSU DATA

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ABSTRACT

The taxonomic placement of selected freshwater and marine *Savoryella* species, as well as *Canalisporium* species originating from submerged wood from aquatic habitats have not been classified into any family or order with certainty. Results based on individual molecular data, analyses of the partial small sequence (SSU data), indicate that *Savoryella* form a monophyletic clade and groups within the subclass Hypocreomycetidae, Sordariomycetes. The genus *Savoryella* shows no affinities with the Hypocreales despite earlier assignment to that order. These findings suggest a new lineage of ascomycetes that have invaded both the sea and freshwater environments. Interestingly, both *Savoryella* and *Ascotaiwania* share few morphological features in common, although clustering in the same clade. Based on morphology and sequence evidence, we suggest their referral to the Hypocreomycetidae incertae sedis, Sordariomycetes, until further information is available.

OBJECTIVES

1. To determine the taxonomic placement of *Savoryella* and *Canalisporium* species based on SSU and LSU sequences.2.To examine the interrelationships of the genera *Savoryella* with the anamophic genus *Canalisporium*.
2. To examine the interrelationships of the genera *Savoryella* with the anamophic genus *Canalisporium*

MATERIAL AND METHODS

The SSU and LSU were amplified and sequenced by using primer NS1, NS3, NS5, NS6, JS1, JS8, LROR, LR7, ITS1, ITS4 and ITS5 (White et al. 1990). Phylogenetic trees were generated from Maximum Parsimony incorporated in PAUP* 4.0b10 (Swofford 2002).

CONCLUSION

1. *Savoryella* species form a monophyletic clade with the anamophic *Canalisporium* species in a sister group (SSU data).
2. *Ascotaiwania* is the teleomorph of *Canalisporium* species (large subunit rRNA gene).
3. The ascomycetes *Savoryella* and *Ascotaiwania* share a common ancestor and differ in some morphological features (28S rDNA gene).
4. The freshwater ascomycetes, previously identified as a *Savoryella* (SS3615) can be assigned to *Ascotaiwania* with the confidence, family a sister group with *Canalisporium* (LSU sequence).

Future work

Further analysis will be focused on wide taxa sampling and sequence of futher genes (ITS, RPB1, RPB2 and EF1-a)

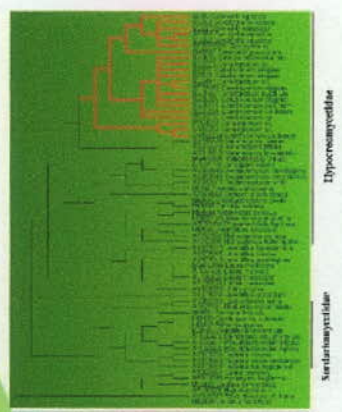

Fig 2 One of 52 parsimonious tree (SSU data)

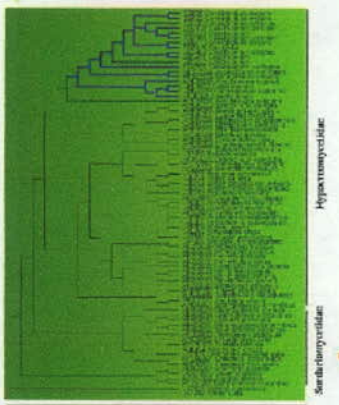

Fig 3 One of 47 parsimonious tree (LSU data)

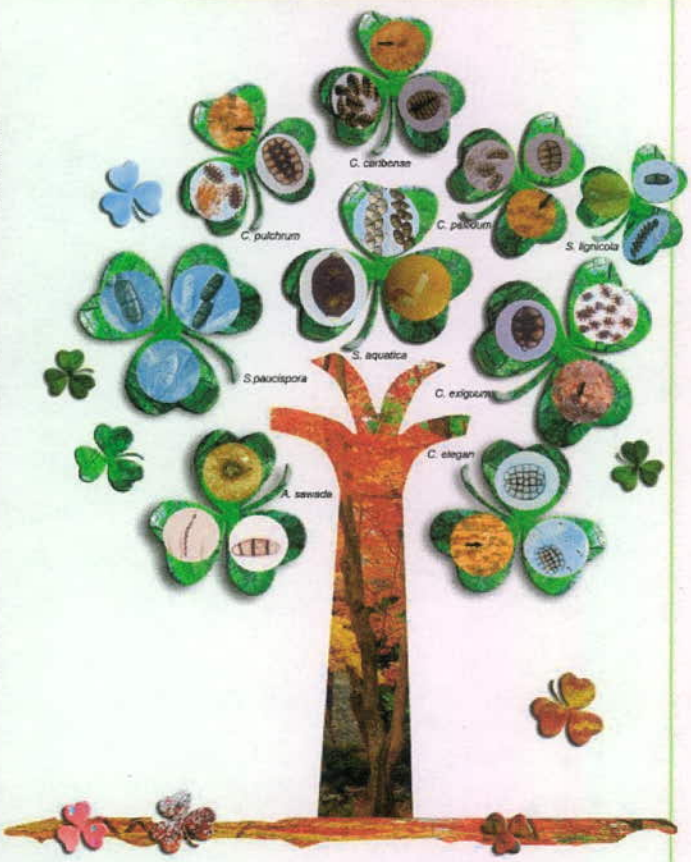

Fig 1 Tree pictures of various spore in this study

REFERENCE

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PART IV

SELECTED FUNGAL SPECIES

PART IV
SELECTED FUNGAL SPECIES

Figure 16 *Canalisporium pulchrum*

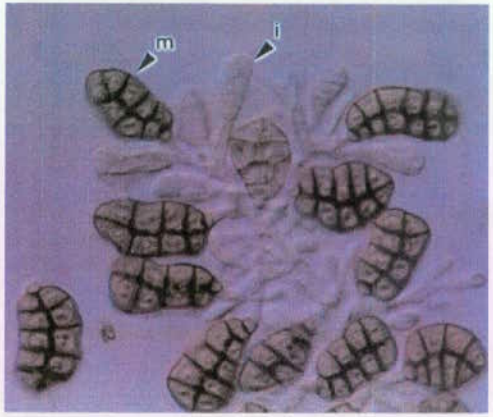

Figure 17 *Canalisporium pallidum*

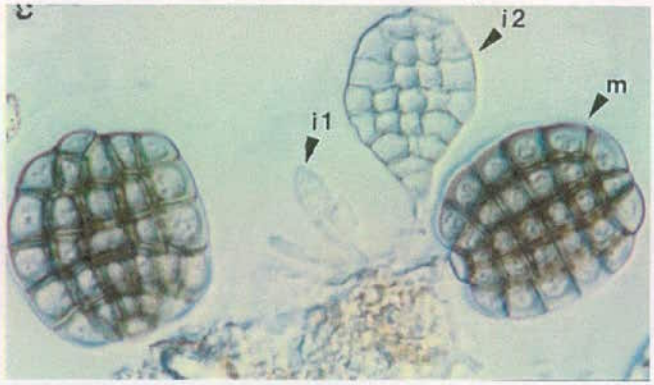

Figure 18 *Canalisporium elegans*

Figure 19 *Canalisporium caribense*

Figure 20 *Savoryella paucispora*

Figure 21 *Savoryella lignicola*

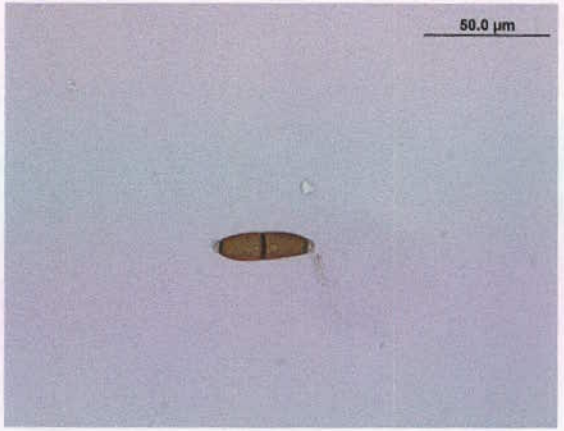

Figure 22 *Savoryella* cf *longispora*

PART V

LIST OF SPECIMENS COLLECTED IN THIS STUDY

PART V

LIST OF SPECIMENS COLLECTED IN THIS STUDY

Table 6. List of *Savoryella* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	Collection Date	IsolationDate	Substrate	SubSite	Site	District	Province	Country
SS00042	3342	-	Sordariales	Incertae sedis	*Savoryella*	*verrucosa*	03-ก.ย.-96	17-ต.ค.-96	Elephant grass	-	Khao Yai National Park	-	Nakhon Ratchasima	Thailand
SS00052	3344	12667	Sordariales	Incertae sedis	*Savoryella*	*verrucosa*	25-ก.ย.-96	29-ต.ค.-96	Twig	-	Khao Yai National Park	-	Nakhon Ratchasima	Thailand
SS00582	3642	-	Sordariales	Incertae sedis	*Savoryella*	*verrucosa*	10-มิ.ย.-98	15-ก.ค.-98	Xylia dolabriformis	Tad Tha Phu	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SAT00320	23612	-	Sordariales	Incertae sedis	*Savoryella*	*longispora*	-	-	Mangrove wood	-	Tammarang Pier	-	Satun	Thailand
SAT00322	23612	-	Sordariales	Incertae sedis	*Savoryella*	*longispora*	-	-	Mangrove wood	-	Tammarang Pier	-	Satun	Thailand
SAT00908	-	-	Sordariales	Incertae sedis	*Savoryella*	*lignicola*	-	-	Mangrove wood	-	Tammarang Pier	-	Satun	Thailand
SAT00866	28374	-	Sordariales	Incertae sedis	*Savoryella*	*paucispora*	-	-	Mangrove wood	-	Laem TaLum Phuk	-	Nakhonsithammarat	Thailand
SAT00867	28375	-	Sordariales	Incertae sedis	*Savoryella*	*paucispora*	-	-	Mangrove wood	-	Laem TaLum Phuk	-	Nakhonsithammarat	Thailand
SS03331	24236	-	Sordariales	Incertae sedis	*Savoryella*	sp.	11-Apr-2005	26-Apr-2005	Stereospermum neuranthum	Tad Tha Phu	Sakaerat Environmental Research Station		Nakhon Ratchasima	Thailand
SS00096	3345	12702	Sordariales	Incertae sedis	*Savoryella*	*aquatica*	14-พ.ย.-96	29-ธ.ค.-96	Anisoptera oblonga	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS00359	3521	-	Sordariales	Incertae sedis	*Savoryella*	*aquatica*	11-ก.ค.-97	06-ส.ค.-97	Alstonia scholaris	Tad Tha Phu	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS00583	3641	-	Sordariales	Incertae sedis	*Savoryella*	*aquatica*	10-มิ.ย.-98	15-ก.ค.-98	Xylia dolabriformis	Tad Tha Phu	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS03801	22509	-	Sordariales	Incertae sedis	*Savoryella*	*aquatica*	26-Jan-2006	15-Mar-2006	Wood		Khao Pra - Bang Khram Wildlife Sanctuary		Krabi	Thailand

Table 7. List of *Ascotaiwania* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	CollectionDate	Isolation Date	Substrate	SubSite	Site	District	Province	Country
SS00051	3343	-	Sordariales	Incertae sedis	*Ascotaiwania*	*sawadae*	25-ก.ย.-96	29-ต.ค.-96	Hard wood	-	Khao Yai National Park	-	Nakhon Nayok	Thailand
SS03615	20507	-	Sordariales	Incertae sedis	*Ascotaiwania*-like	-	-	-	Submerged *Wrightia*	Khlong I-Gading stream	Hala-Bala Wildlife Sanctuary		Narathiwat	Thailand

Table 8. List of *Monotosporella* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	CollectionDate	Isolation Date	Substrate	SubSite	Site	District	Province	Country
SS01013	9964	-	Xylariales	Amphisphaeriaceae	Monotosporella	sp.	18-Oct-2001	15-Jan-2001	Wood	-	Mu Ko Chang National Park	-	Trat	Thailand
SS01025	9953	-	Xylariales	Amphisphaeriaceae	Monotosporella	sp.	27-Sep-2000	22-Jan-2001	Wood	Tor Tip Waterfall	Kaeng Krachan National Park	-	Prachuap Khiri Khan	Thailand

Table 9. List of *Canalisporium* strains used in this study

Original Code	BCC Code	BBH Code	Order	Family	Genus	Epithet	CollectionDate	Isolation Date	Substrate	SubSite	Site	District	Province	Country
SS00091	3350	12699	Incertae sedis	Incertae sedis	*Canalisporium*	*pallidium*	14-พ.ย.-96	27-ธ.ค.-96	Alstonia scholaris	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS00170	3406	12764	Incertae sedis	Incertae sedis	*Canalisporium*	*pulchrum*	02-ธ.ค.-96	11-ก.พ.-97	Alstonia scholaris	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS00498	3608	-	Incertae sedis	Incertae sedis	*Canalisporium*	*pallidium*	06-พ.ย.-97	09-มี.ค.-98	Xylia dolabriformis	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS00523	3625	-	Incertae sedis	Incertae sedis	*Canalisporium*	*elegans*	14-เม.ย.-98	24-เม.ย.-98	Xylia dolabriformis	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand

SS00809	12770	-	Incertae sedis	Incertae sedis	*Canalisporium*	*exiguum*	20-ก.ย.-00	29-ก.ย.-00	Wood		Khao Soi Dao Wildlife Sanctuary	-	Chanthaburi	Thailand
SS00877	9963	-	Incertae sedis	Incertae sedis	*Canalisporium*	*elegans*	26-ก.ย.-00	10-พ.ย.-00	Wood	Road marker at km 18	Kaeng Krachan National Park	-	Prachuap Khiri Khan	Thailand
SS00895	12772	-	Incertae sedis	Incertae sedis	*Canalisporium*	*elegans*	13-พ.ย.-00	29-พ.ย.-00	Stereospermum neuranthum	Road marker at km 29.2	Sakaerat Environmental Research Station	-	Nakhon Ratchasima	Thailand
SS03483	26225	-	Incertae sedis	Incertae sedis	*Canalisporium*	*elegans*	8-Apr-2005	17-Jul-2005	Wood	-	Bor Kleng Hot Spring	-	Ratchaburi	Thailand
SS03491	18364	-	Incertae sedis	Incertae sedis	*Canalisporium*	sp.	8-Apr-2005	19-Jul-2005	Wood	-	Kaeng Krachan National Park		Prachuap Khiri Khan	Thailand
SS03683	21022	-	Incertae sedis	Incertae sedis	*Canalisporium*	sp.	13-Jul-2005	7-Feb-2006	Wood	Wang Kar Leung Waterfall	Wang Kan Lueng Arboretum		Lop Buri	Thailand
SS03732	21424	-	Incertae sedis	Incertae sedis	*Canalisporium*	sp.	17-May-2004	21-Feb-2006	Wood	Ban Krang	Kaeng Krachan National Park		Phetchaburi	Thailand
SS03773	21030	-	Incertae sedis	Incertae sedis	*Canalisporium*	sp.	28-Jan-2006	10-Mar-2006	Leaf	Khlong I-Gading	Hala-Bala Wildlife Sanctuary		Narathiwat	Thailand
SS03788	22507	-	Incertae sedis	Incertae sedis	*Canalisporium*	sp.	26-Jan-2006	15-Mar-2006	Wood	-	Khao Pra - Bang Khram Wildlife Sanctuary		Krabi	Thailand
SS03819	21221	-	Incertae sedis	Incertae sedis	*Canalisporium*	*pulchrum*	26-Jan-2006	15-Mar-2006	Wood	-	Khao Pra - Bang Khram Wildlife Sanctuary		Krabi	Thailand
SS03823	21428	-	Incertae sedis	Incertae sedis	*Canalisporium*	*pulchrum*	26-Jan-2006	15-Mar-2006	Wood	-	Khao Pra - Bang Khram Wildlife Sanctuary		Krabi	Thailand
SS03839	24239	-	Incertae sedis	Incertae sedis	*Canalisporium*	*caribense*	25-Feb-2006	17-May-2006	Wood	Khlong I-Gading	Hala-Bala Wildlife Sanctuary		Narathiwat	Thailand

PART VI

DRAFT MANUSCRIPT FOR PUBLICATION IN MYCOLOGICAL RESEARCH

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The phylogenetic relationship of the genera *Ascotaiwania*, *Savoryella* (Hypocreomycetidae *incertae sedis*, Sordariomycetes, Ascomycota) and the anamorphs *Canalisporium* and *Monotosporella,* as inferred by SSU, LSU, ITS and RPB2 data

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ABSTRACT

The taxonomic placement of freshwater and marine *Savoryella* species has been widely debated and the genus assigned tentatively to various orders in the *Sordariomycetes*. Results based on molecular data analyses of the partial small subunit rRNA (SSU), large subunit rRNA (LSU), internal transcribed spacer rDNA (ITS) and combined SSU_LSU_RPB2 sequences indicates that *Savoryella* species form a monophyletic clade, with *Ascotaiwania*, and its anamorphs, in a sister clade. *Savoryella* shows no affinities with the Hypocreales, Halosphaeriales, Sordariales and

Xylariales, despite earlier assignments to those orders. As the genus shows no relationship with any order or family, it is best referred to the Hypocreomycetidae *incertae sedis*, Sordariomycetes, until further information is available. Anamorphs of *Ascotaiwania* include *Monotosporella, Helicoon* while *Canalisporium* species are reported for the first time as anamorphs of the genus.

Key words: *Ascotaiwania, Canalisporium, Monotosporella, Savoryella*, phylogeny, systematics.

Introduction

Savoryella is one of the most commonly reported unitunicate ascomycete genus from submerged wood in rivers or streams (Sivichai et al., 2002, 2003) and the marine environment (Jones & Hyde, 1992), while *S. appendiculata* and *S. melanospora* have been recovered from wood in contact with sand (Jones & Hyde, 1992; and Abdel-Wahab and Jones, 2000). The phylogenetic assignment of the genus is unresolved and it has been referred to a number of orders and families in the Sordariomycetes, Sordariomycetidae (Zhang et al., 2006). Eleven species, *Savoryella appendiculata, S. aquatica, S. curvispora, S. fusiformis, S. grandispora, S. lignicola, S. limnetica, S. longispora, S. melanospora, S. paucispora* and *S. verrucosa*, are recognized of which five are marine, while the remainder are found in freshwater habitats (Cai et al., 2006).

This genus was established by Jones and Eaton (1969) with *S. lignicola* as the type species, from wooden slats in a water cooling tower run on brackish water. It is characterized by dark brown to black ascomata, clavate to cylindrical asci with a comparatively flattened apical ring and veriscolourous septate ascospores, brown central and hyaline end-cells. No anamorph has been reported for *Savoryella.* The genus has been variously referred to Sphaeriales *incertae sedis* (Kohlmeyer and Kohlmeyer, 1979), Ascomycetes *incertae sedis* (Kohlmeyer, 1986; Eriksson and Hawksworth, 1986), Amphisphaeriaceae (Eriksson and Hawksworth, 1987) and Sordariales (Jones and Hyde, 1992). Barr (1990), on the basis of its morphological features (catenophyses-like paraphyeses) ultrastructural observations (Read et al., 1993) considered it best referred to the Halosphaeriales. Recently, based on LSU rDNA data, Vijaykrishna (2006) and Cai et al. (2006) accommodated two species (*S. elongate* and *S. longispora*) in the Hypocreales within the Hypocreomycetidae, but its relationship with other orders could not be elucidated with good statistical support.

Ascotaiwania (Sivanesan and Chang, 1992) morphologically resembles *Savoryella* with its versicolourous ascospores but differs in having cylindrical asci with a relatively massive, non-amyloid apical ring, ascospores that are 4-8-septate, and anamorphs in *Monotosporella* (*Ascotaiwania sawadae, A. mitriformis*) and *Helicoon farinosum* (*A. hughesii*) (Sivichai *et al.*, 1998; Cai *et al.*, 2006). A molecular study has failed to resolve the taxonomic position of *Ascotaiwania* (Ranghoo *et al.*, 1999) with Cai *et al.* (2006) referring it to the Sordariales *incertae sedis*.

In our ongoing research of Thai freshwater fungi (Sivichai et al., 2002, 2003; Pang et al., 2002; Pinruan et al., 2002, 2004a, 2004b; Pinoi et al., 2003) a number of *Canalisporium* species have been recovered from submerged or trapped wood (Sivichai and Boonyene, 2004). One species was always associated with a new species of *Ascotawania*, and both were isolated into axenic culture. Cultures derived from ascospores yielded a *Canalisporium elegans*, establishing a third anamorph for the genus *Ascotawania.* Currently nine *Canalisporium* species have been described (*Canalisporium caribense*, *C. elegans*, *C. exiguum*, *C. jinghongensis, C. kenyense*, *C. pallidum*, *C. panamense*, *C. pulchrum* and *C. variabile*), all from freshwater habitats. Cai et al. (2006) consider *Canalisporium* as anamorphic Tubeufiaceae, Pleopsorales.

The objective of this study is to determine: 1. The monophyly of the genera *Ascotawania, Canalisporium* and *Savoryella*, 2. The phylogenetic relationship of *Ascotawania* and *Savoryella*, and 3. The familial and ordinal status of these two genera, both currently classified as Ascomycetes *incertae sedis*.

Materials and methods

Specimen collection

Fungi were isolated from various substrata from freshwater and marine locations in Thailand (Sivichai and Boonyene, 2004; Sakayaroj et al., 2004; Pinruan et al., 2002) and maintained on CMA or PDA media with seawater or freshwater.

Fungal isolates and growth

Fungal cultures were deposited and maintained in the BIOTEC Culture Collection (BCC) and taxa used in this study are listed Table 1. All cultures were grown on potato dextrose agar (PDA) at room temperature of 25°C for 4-16 weeks (depending on the growth rate of each species).

Genomic extraction and PCR amplification

Actively growing mycelia were scraped off the surface of a culture and transferred to micro-centrifuge tubes and the biomass were lyophilized at -80°C for 2 days before DNA extraction which followed a modified protocol of Tigano-Milani et al (1995). The lyophilized-mycelia were ground with a sterile pipette tip in 2 ml microcentrifuge tube. The resulting powder was transferred to a 1.5-mL pre-warmed (65°C) microcentrifuge tube with 700 µl extraction buffer (0.7 M NaCl; 50 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; 1% CTAB) and incubated at 65°C for 1 hour. In the CTAB-based method, DNA was extracted once with 500 µl (24:1) chloroform-isoamyl alcohol (CIAA) and centrifuged at 12.000 rpm for 20 minutes. The supernatant was transferred to a 1.5-mL new microcentrifuge tube containing 1/10 volume of 10% CTAB, added with 700 µl CIAA and centrifuged for 20 minutes at 12.000 rpm. The 1000 µl precipitation buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% CTAB) were added to the aqueous phase of supernatant for 30 minutes at room temperature. The 300 µl Tris-EDTA High Salt (1 M NaCl; 10 mM EDTA, pH 8.0; 1 mM EDTA, pH 8) buffer were added to the pellet, washed with 400 µl ethanol 70%, and resuspended in 30 µL sterilized deionized water containing 5 µ RNase A (100 µg/mL). The DNA pellet after centrifugation (20 minutes, 12.000 rpm, 4 °C) was washed in 400 µl 70% ethanol and air-dried. Finally, the DNA was re-suspended in 50 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0).

The partial SSU, LSU ribosomal DNA, ITS region and partial RPB2 were amplified using primers NS1, NS3, NS4, NS5, NS6, JS1, JS8, LROR, LR5, LR7, ITS1, ITS4, ITS5, RPB2-5F2 and RPB2-7CR (White et al 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al 1999). PCR reactions were carried out in total volume of 50 µl containing 10-50 ng DNA template. The 50 10X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs, 0.2 µM each primer and 0.5 U of Taq Polymerase (DNA Polymerase Kit, Vivantis Technologies). Amplification cycles were performed following the procedure of Tang et al (2007) composed of 95°C for 5 min, followed by denaturation step at 35 cycles, 52°C for 1 min (for SSU or LSU rDNA), 55°C for 1.5 minute (ITS region), 55°C for 1.5 minute (for RPB2) at annealing step, 72°C for 1.5 minutes (elongation step) and the final step of 72°C for 10 minutes. The size of each amplified

fragment was verified by gel electrophoresis with ethidium bromide staining of a 2 mL product sample and visualized over an ultraviolet transilluminator. PCR products were purified using NucleoSpin^R Extract Kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Then checking for the quantity and quality in a 1% agarose gel electrophoresis was applied. Finally, the purified PCR product was used directly for DNA sequencing.

DNA sequencing

PCR products were directly sequenced by the Macrogen., Inc in Korea using forward and reverse primers with the same primers (White et al 1990; Bunyard et al., 1994; Landvik, 1996; Liu et al 1999). Each sequence was checked for ambiguous bases and assembled using Bioedit 7.5.03 (Hall, 2006).

Sequence alignment and phylogenetic analyses

A BLAST search was employed to obtain the closest matched sequences in the GenBank database (Altschul et al., 1990). The SSU, LSU, ITS rDNA and RPB2 sequences were multiple aligned along with other related sequences obtained from GenBank (Zhang et al 2006; Tang et al 2007; Schoch et al 2007) using Clustal W 1.6 (Thompson et al., 1994). The result was further adjusted manually to allow for maximum alignment using BioEdit 7.5.0.3 (Hall, 2006). Gaps were always coded as missing data. Regions in which alignment was ambiguous due to the large number of gaps were deleted from the analysis. *Daldinia concentrica* and *Xylaria hypoxylon* were chosen as the outgroup taxa for all analyses.

The aligned dataset was subsequently analyzed using maximum parsimony (MP) in PAUP 4.0b10 (Swofford, 2002), for the most parsimonious trees (MPTs). Heuristic searches algorithm with tree bisection–reconnection (TBR) branch swapping, 1000 replicates of random stepwise sequence addition, were performed. Gaps were treated as missing data and given equal weight. The Kishino–Hasegawa (K–H) test was used for estimation of the best tree topology (Kishino and Hasegawa, 1989). Bootstrapping analyses (Felsenstein 1985) were performed with full heuristic search on 1000 replicates (10 replicates of random-swanpping algorithm). The tree length (TL), Consistency indices (CI), and Retention indices (RI) were calculated for

each tree generated. Sequences representative of the different orders within the class Sordariomycetes were retrieved from Genbank and added to the alignment

Results

SSU phylogeny

To determine the taxonomic position and investigate the monophyly of the genera *Ascotaiwania, Canalisporium* and *Savoryella* at the ordinal level, the type species of *Savoryella* (*S. lignicola*) and *Canalisporium* (*C. carebense*) were also included in the 18S rDNA dataset. Thirty–two taxa of *Ascotaiwania, Canalisporium* and *Savoryella* from the BIOTEC Culture Collection (BCC) were aligned along with representative taxa from Class Sordariomycetes with three main Subclasses: Hypocreomycetidae, Sordariomycetidae and Spathulosporomycetidae. In subclasse Hypocreomycetidae, various taxa from four orders, consisting of the Halosphaeriales, Microascales, Hypocreales, Melanosporales and Hypocreomycetidae *incertae sedis* (unnamed clade) were included in the analysis, whereas seven major orders from the Subclasse Sordariomycetidae (Diaporthales, Coniochaetales, Chaetosphaeriales, Calosphaeriales, Ophiostomatales, Sordariales and Boliniales) and two taxa of the ascomycetes *incertae sedis* (*Pseudohalonectria falcata* and *P. falcate*) were incorporated with this study. Members of the order *Xylariales* (*Daldinia concentrica* and *Xylaria hypoxylon*) were chosen as the outgroup taxa for this data.

Maximum parsimony resulted in 18 most parsimonious trees (MPTs) with tree length (TL) 2309 steps, Consistency indices (CI) and Retention indices (RI), Homoplasy indices, respectively. Initial analysis of this dataset with a tree length of 2309 (CI=0.472, RI=0.846, RC= 0.400, HI=0.528) shown in Figure 3. A total of 1189 characters, 532 are parsimony informative, 497 are constant characters, 160 are variable character (parsimony uninformative).

The genera *Savoryella*, *Canalisporium* and *Ascotaiwania* formed a well supported clade (ACS clade) and clearly distinct from the Halosphaeriales, Hypocreales, Melanosporales, Miciroascales (Hypocreomycetidae) and Sordariales (Sordariomycetidae).

The four *Canalisporium* species (*C. caribense*, *C. elegans*, *C. pallidum* and *C. pulchrum*) and five *Savoryella* species (*S. aquatica*, *S. lignicola*, *S. longispora*, *S.*

paucispora and *S. verrucosa*) formed a monophyletic subclade with a well-supported bootstrapping (Figures 3-4).

The *Ascotaiwania-like* sp. nov (SS03615 or BCC20507) grouped with the *Canalisporium* species, but this relationship did not receive any support. However, *C. exiguum* formed a basal clade to the the *Savoryella* subclade, with low support (76%).

LSU phylogeny

This 28S rDNA dataset is to investigate the phylogenetic relationship of the genera *Savoryella* (five sequences from Thai marine isolates and eight sequences from Thai aquatic isolates), *Ascotaiwania*-like sp. nov. (SS03615), *Canalisporium* (17 sequences) and *A. sawadae* (SS00051).

Five sequences from the GanBank (*Monotosporella setosa* AF132334, *A. hughesii* AY316357, *A. sawadae* AF132323, *A. mitriformis* AF132324 and *A. persoonii* AY590295) were also added to this analysis with two *Xylaria* species as the outgroup. The number of most parsimonious trees (MPT), tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) are listed in Figure 10. Total of a 1241 characters, 289 are parsimony informative, 812 are constant characters.

The Kishino–Hasegawa (K–H) test was used for estimation of the best tree topology MP analysis shown in Figure 10. The tree originated by unweighted parsimony analysis yields the best KH-likelihood scores shown in the Figure 10. All topologies are similar to the phylogeny generated from the ITS dataset (data not shown). According to our analyses, our sequences based on the LSU rDNA data are divided into at least three major clades. Representative clades with bootstrap support values (BS) above 50% were designated as follows:

Clade A (*Savoryella* clade): *S. lignicola* (SAT00908), *S. longispora* (SAT00320, (SAT00322), *S. paucispora* (SAT00866, SAT00867), *S. veruscosa* (SS00042), *S. aquatica* (SS00096, SS00583, SS00359, SS03801), *Savoryella* cf *verucosa* (SS03331) and *S. verrucosa* (SS00582, SS00052). The clade is composed of two distinct groups of species (A1: marine-derived *Savoryella* species and A2: freshwater-derived *Savoryella* species); both are characterized by their habitat origin. Most of the internal nodes of each clade have moderate to high bootstrap support (51-100%) indicating that within each group, they are closely related. Within this clade,

the first group of the marine species (A1) were represented by *S. lignicola* (SAT00908), *S. longispora* (SAT00320, SAT00322), *S. paucispora* (SAT00866, SAT00867), while the second group (A2) comprises two species of *Savoryella* (*S. aquatica* and *S. verrucosa*) originate in a freshwater environment collected from submerged wood.

Clade B *Canalisporium* consist of *Ascotaiwania*-like sp. nov. (SS03615), *C. elegans* (SS00877), *C. pulchrum* (SS03819, SS03823, SS03982, SS00170, SS03788), *Canalisporium* cf *pulchrum* (SS03773), *C. elegans* (SS00523, SS00895, SS03483), *Canalisporium* cf *elegans* (SS03491), *Canalisporium* sp. (SS03732), *C. exiguum* (SS00809), *C. caribense* (SS03839), *Canalisporium* cf *caribense* (SS03683) and *C. palladium* (SS00091, SS00498).

The *Canalisporium* species are considered monophyletic, but again divide into 2 groups: B1 comprises most of the speices while *C. palladium* forms a sister group with high support. *Ascotaiwania* species do not form a monophyletic clade. *A. hughesii* and its anamorph formed a sister group to the *Savoryella/Canalisporium* clades, while *A. sawadae* and *A. mitriformis* formed a separated clade to the *Savoryella/Canalisporium* clade.

Clade C "*Ascotaiwania*" spp., comprise *A. sawadae* (SS00051), *M. setosa* (AF132334), *A. hughesii* (AY316357), *A. sawadae* (AF132323), *A. mitriformis* (AF132324) and *A. persoonii* (AY590295), form a sister group with Clade A and B. Most taxa are sequences derived from the GenBank. Within this Clade, *A. persoonii* (AY590295) is basal to all other taxa but without any support (subclade C3). The grouping of *A. sawadae* (SS00051) and *A. sawadae* (AF132323) is 100%, while *A. mitriformis* (AF132324) forms as a basal sister taxon (bootstrap values= 84%) in subclade C2, with other taxa *M. setosa* (AF132334) and *A. hughesii* (AY316357) in the subclade C1 with a weak support (bootstrap values= 53%). Within subclade C1, *M. setosa* (AF132334) and *A. hughesii* (AY316357) are closely related with high bootstrap support.

In this study, *Savoryella* species and *Canalisporium* species form a monophyletic groups (within the subclass Hypocreomycetidae, the Class Sordariomycetes), with *Ascotaiwania* spp. as a sister clade. The exception is *Ascotaiwania*-like sp. nov (SS03615).

ITS phylogeny

The ribosomal (ITS1, 5.8S, ITS2) sequence dataset was analyzed by parsimony analysis. The resulting dataset comprised 35 sequences; with *Xylaria hypoxylon* (FJ205468) and *Daldinia concentrica* as the outgroup taxa. Initial analysis of this dataset yielded 46 trees with a tree length (TL) of 1693 (CI= 0.615, RI= 0.808, RC=0.497, HI=0.385) shown in Figure 11. A total of 758 characters, 491 are parsimony informative and 196 are constant characters.

In the analysis of the ITS sequence (the genera *Canalisporium* and *Savoryella*) showed a common node with the bootstrap (67%). Fifteen *Canalisporium* formed a well-supported monophyletic clade strongly support by 100% bootstrap with *Savoyella* species grouped as a siter clade. The two *A. sawadae* strains were monophylytic with 85% bootstrap support. Twelve *Savoryella* species constitute a well-supported monophyletic clade with a bootstrap value of 97% and appeared to be phylogenetically distinct from other genera such as *Canalisporium*, *Monotosporella*, *Ascotaiwania* and *Helicoon* (Figure 11). Within the *Savoryella* clade, most of the internal subclades did not receive reliable branch support. The Thai marine strains *Savoryella* cf *longispora* (SAT00320) and *S. paucispora* (SAT00866, SAT00866) grouped together, but with weak statistical confidence. However, the position of *S. lignicola* (SAT00908), the type species and a marine isolate, did not cluster with other *Savoryella* derived from marine habitats. Instead, it was basal to other Thai freshwater *Savoryella* species. In the Thai freshwater *Savoryella* subclade, four isolates of *S. aquatica* group consistently with 85% bootstrap support, while *S. verrucosa* clusters separately in this subclade with 86% bootstrap support. *Monotosporella* strains and two *Helicoon* strains did not group with the *Ascotaiwania* strains.

The congruence of ITS rDNA and LSU rDNA datasets derived phylogenies was tested by analyzing the respective dataset independently with both Bayesian (data not shown) and parsimony. Separated parsimony phylogenetic analyses of the ITS region dataset and partial LSU dataset resulted in similar topologies, both data providing better resolution of deeper nodes.

Combined SSU LSU RPB2

The combined SSU+LSU+RPB2 dataset (based on maximum parsimony analysis) was computed with the SSU+GenBank, individual LSU, the ITS, combined

SSU+LSU, RPB2, the combined ITS+RPB2 and the ITS+LSU rDNA datasets, in order to compare the tree topology.

The sequence data in this analysis as a combined dataset consisted of 3369 characters, 1053 are parsimony informative, 390 were variable (parsimony uninformative) and 1926 were constant. Initial analysis of this dataset yielded 8 trees with a tree length of 3528 (CI= 0.613, RI= 0.803, RC= 0.492, HI=0.387 shown in Figure 15.

Ascotaiwania strains are polyphyletic with *A. sawadae* and *A. mitriformis* as a sister group to the *Canalisporium/Savoryella* clades, but with weak support. *Ascotaiwania hughesii* and *A. persoonii* formed separate clades to the *Canalisporium/Savoryella* clades. *Canalisporium* strains formed a monophyletic group with *A. sawadae* as a sitster clade. Five *Savoryella* species (*S. aquatica*, *S. lignicola*, *S. longispora*, *S. paucispora* and *S. verrucosa*) formed a monophyletic subclade with high bootstrap support.

DISCUSSION

A new lineage of the ACS clade

Hibbett et al (2007) accepted three subclasses in the Sordariomycetes: Hypocreomycetidae (with the orders Coronophorales, Hypocreales, Melanosporales, Microascales); Sordariomycetidae (with the orders Boliniales, Chaetosphaeriales, Coniochaetales, Diaporthales, Ophiostomatales, Sordariales) and the Xylariomycetidae (with the order Xylariales), while the orders Lulworthiales, Meliolales, Phyllachorales and Trichosphaeriales are represented as Sordariomycetes *incertaesedis*.

The genera *Ascotaiwania*, *Canalisporium* and *Savoyella* studied here formed a clade (here after referred to as ACS) within the Hypocreomycetidae with the Coronophorales and the TBM clade as sister clades. They form a distinct clade to the order Halosphaeriales, Microacales and Hypocreales, whereas genera grouping in the TBM clade are morphologically diversed to those in the ACS clade. The ACS clades have a numbers of shared features: ascomata generally swan-like shaped rarely with a central neck, unitunicate asci, that are persistent, clavate to cylindrical, short pedunculate with without paraphyses, generally with an apical pore, ascospores, asci

cells, cell hyphae-like, central cells brown. Most ascospore appendages are lacking except for the marine species of *S. appendiculata.*

All are saprobes; most are aquatic and well growing on decayed wood as lignocellulose materials (Sivichai et al., 2002, 2003). However, few are active degrades of lignicelluose (Jones & Eaton 1969). The ACS clade represents yet another new lineage of the Hypocreomycetidae. It is interesting that both the TMB and ACS clades occur in aquatic habitats, transitional from tesrestrial to freshwater to brackish and fully saline habitats.

Although the ACS clade represents a new lineage of ascomycetes, it is premature to elect a new order to accommodate this group of taxa.

No anamophs have been reported for *Savoyella*, while several and dematiaceous hyphomycetes have been reported to the genus *Ascotaiwania*: *Monotosporella* sp. (*A. sawadae*; Sivichai et al., 1998), *M. setosa* (*A. sawadae*; Ranghoo et al, 1999) and *Helicoon* (*A. hughesii*; Fallah et al., 1999; Tsui and Berbee, 2006). In our analyses, *Ascotaiwania* is not monophyletic, although they form a distinct group (Ranghoo et al, 1999; Cambell and shearer, 2004).

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PART VII

THAI ARTICLE SENT TO BRT MAGAZINE

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การศึกษาจัดจำแนกในระดับโมเลกุลของราสกุล *Savoryella* และ *Canalisporium* โดยใช้เทคนิคทางชีวโมเลกุลของยีนหลายชนิดร่วมในการจัดหมวดหมู่

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ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ อุทยานวิทยาศาสตร์ 113 ถนนพหลโยธิน ตำบลคลองหนึ่ง อำเภอคลองหลวง จังหวัดปทุมธานี 12120

การศึกษาค้นคว้าและวิจัยงานอนุกรมวิธานทางด้านราวิทยา(Mycology) มีความจำเป็นอย่างยิ่งในการต่อยอดองค์ความรู้ในงานทางด้านต่างๆเพื่อนำไปใช้ประโยชน์ทางเทคโนโลยีชีวภาพและพันธุวิศวกรรมในสาขาที่เกี่ยวข้อง ราจัดเป็นจุลินทรีย์ในอาณาจักรรา (Kingdom of Fungi) หรืออาณาจักรรา มีหน้าที่และบทบาทในระบบนิเวศน์ที่สำคัญอย่างยิ่งในการย่อยสลายอินทรีย์วัตถุ (Saprobes) เพื่อนำแร่ธาตุต่างๆกลับคืนสู่ธรรมชาติ โดยราถูกนำมาใช้ประโยชน์ต่างๆอย่างมากมายในชีวิตประจำวัน ทั้งด้านเกษตรกรรม และด้านอุตสาหกรรม ราบางชนิดนำมาใช้เป็นอาหารเช่น เห็ดที่รับประทานได้ชนิดต่างๆ ราบางกลุ่มถูกใช้ในขบวนการผลิตส่วนประกอบของอาหารเช่น ซีอิ้ว เต้าเจียว เนยแข็ง เป็นต้น อีกทั้งราบางชนิดมีความสามารถในการสร้างสารออกฤทธิ์ทางชีวภาพซึ่งเป็นแนวทางการพัฒนาและสร้างยาใหม่ๆในอนาคตเพื่อใช้ประโยชน์ทางการแพทย์

เนื่องจากรามีความหลากหลายทางชีวภาพ (Fungal biodiversity) ทั้งในแง่สกุล (Genera) ชนิด (Species)และสายพันธุ์ (Varieties) ดังนั้นการนำราไปใช้ประโยชน์ในด้านต่างๆจึงจำเป็นต้องทราบข้อมูลทางด้านอนุกรมวิธาน ซึ่งในการจัดกลุ่มราต้องอาศัยการศึกษาควบคู่กันระหว่างเทคนิคทางด้านลักษณะทางสัณฐานวิทยา (Morphological study) และเทคนิคการศึกษาเชิงลึกในระดับโมเลกุล (Molecular study) เพื่อทำให้ทราบระดับอนุกรมวิธานที่ชัดเจนก่อนที่จะนำไปใช้ประกอบการศึกษาวิจัยในด้านต่างๆที่เกี่ยวข้องต่อไป งานทางด้านราวิทยาถือว่าเป็นงานวิจัยทางด้านพื้นฐานทั้งช่วยสนับสนุนงานวิจัยทางด้านการใช้ประโยชน์ให้มีประสิทธิภาพยิ่งขึ้น

หลักการจำแนกกลุ่มรานักราวิทยาจะพิจาณาภาพรวมจากกลุ่มใหญ่ไปยังกลุ่มย่อย ซึ่งเรียกว่า “แทกโซโนมี” (Taxonomy) มีข้อมูลพื้นฐานดังนี้ “ไฟลัม” (Phylum) “คลาส” (Class) “ออเดอร์หรือระดับ” (Order) “แฟมีลี่ หรือ วงศ์” (Family) “จีนัสหรือสกุล” (Genus) “สปีชีสหรือชนิด” (Species) ชนิด และ “สายพันธุ์ หรือ สเทนส์” (varieties/strains)

การจัดจำแนกโดยอาศัยข้อมูลลำดับเบส rDNA ถือได้ว่ามีความแม่นยำสูงมากเมื่อเปรียบเทียบกับการจัดจำแนกโดยอาศัยลักษณะทางสัณฐานวิทยาของราเพียงอย่างเดียวซึ่งใช้

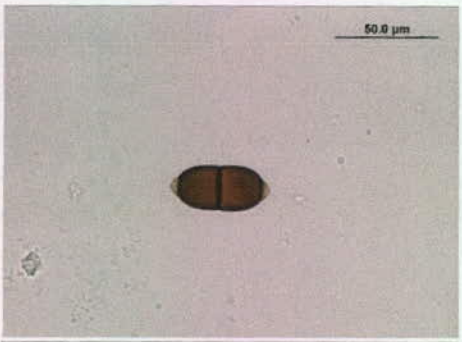
โครงสร้างต่างๆและส่วนสืบพันธุ์ของราในการจัดจำแนก เนื่องจากอาจมีข้อจำกัดบางประการในการจัดจำแนกอาศัยลักษณะทางสัณฐานวิทยาของราแต่ละชนิดซึ่งอาจมีความความผันแปรเกิดขึ้นได้ ตามสภาพแวดล้อมที่เปลี่ยนไป จึงทำให้ยากในการการจัดหมวดหมู่กลุ่มราต่างๆ ถึงแม้นักราวิทยาจะมีความเชี่ยวชาญในรากลุ่มดังกล่าวก็ตาม

ราสกุล *Savoryella* มีรูปร่างของสปอร์ที่เกิดจากการสืบพันธุ์แบบอาศัยเพศ (Teleomorph) พบได้ทั้งน้ำจืดและน้ำทะเล มีหน้าที่และบทบาทในระบบนิเวศน์ในฐานะเป็นผู้ย่อยสลายอินทรีวัตถุทั้งใบไม้ และ ไม้ที่ย่อยสลาย (Saprobes) จากการศึกษาเบื้องต้นพบว่าข้อมูลทางด้านสัณฐานวิทยาของราดังกล่าวมีความคลุมเครือและในการจัดจำแนกในระดับ ออเดอร์ ซึ่งเคยถูกรายงาน ตามการจำแนกโดยการใช้สัณฐานวิทยาเท่านั้น ผลการศึกษาดังกล่าวยังมีความไม่ชัดเจนในการจัดกลุ่มโดยเฉพาะบทความวิจัยต่างๆ ในการจัด "ออเดอร์" ที่ชัดเจนใน ออเดอร์ Sordariales, ออเดอร์ Halosphaeriales และ ออเดอร์ Hypocreales ซึ่งทั้งสาม "ออเดอร์" อยู่ในซับคลาส Hypocreomycetidae รวมทั้งข้อมูลเบื้องต้นพบว่าจากการสังเกต ราน้ำจืด สกุล *Savoryella* บางสายพันธุ์มีการเจริญเติบโตควบคู่กันและมักพบพร้อมกันเสมอบนไม้ที่ย่อยสลาย ของ ราสกุล *Canalisporium* บางสายพันธุ์ซึ่งมีโครงสร้างการสืบพันธุ์ แบบไม่อาศัยเพศ (Anamorph).

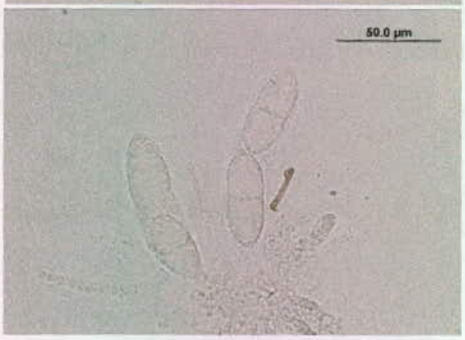
การศึกษาครั้งนี้ ใช้การหาความสัมพันธ์เชิงวิวัฒนาการในระดับโมเลกุล (Molecular Phylogeny) มาช่วยตอบคำถาม และช่วยหา ตำแหน่งของอนุกรมวิธานระดับโมเลกุลของราดังกล่าว โดยใช้ ข้อมูลในลำดับเบสบริเวณของไรโบโซมอลอาร์เอ็นเอชนิด 18S (SSU) และ ไรโบโซมอลอาร์เอ็นเอชนิด 28S (LSU) เป็นยีนที่ช่วยจัดกลุ่มและจำแนกในระดับ คลาส ออเดอร์ และแฟมีลี่ หรือ วงศ์ได้อย่างกว้างๆได้ดี ในขณะที่ ดีเอนเอของลำดับเบสในบริเวณไอทีเอส 1 และ 2 รวมกับไรโบโซมอลอาร์เอ็นเอ ชนิด 5.8S (ITS1-2, 5.8S) และ อาร์พีบีทู (RPB2) สามารถบ่งชี้ใน ระดับสกุลและ"สปีชีสหรือชนิด" (Species) ชนิด ได้ดีที่สุด รวมทั้งจะช่วยยืนยันผลของ SSU และ LSU ด้วย ตามการรายงานในหลายบทความในงานทางด้านราวิทยา

ผลการศึกษาในการจำแนกโดยใช้เทคนิคในระดับโมเลกุลพบว่าผลการจัดกลุ่มในระดับโมเลกุลของ ราสกุล *Savoryella* ให้ผลที่ขัดแย้งกับผลการจัดจำแนกโดยอาศัยลักษณะทางสัณฐานวิทยาของรา และ พบว่า การจัดจำแนกในระดับโมเลกุลในยีนหลายชนิดให้ผลไปในทิศทางเดียวกัน คือ ราสกุล *Savoryella* ไม่ได้ถูกจัดในออเดอร์ (order) ดังกล่าว และ ข้อมูลของ 5.8S (ITS1-2, 5.8S) และ อาร์พีบีทู (RPB2) พบว่า ราสกุล *Savoryella* กับ ราสกุล *Canalisporium* มีความใกล้เคียงกันอย่างมากและมีบรรพบุรุษร่วมกัน ตาม "แผนภูมิวิวัฒนการ" หรือ แผนภูมิ การหาความสัมพันธ์เชิงวิวัฒนาการในระดับโมเลกุล เป็นเครื่องมือในการศึกษาวิวัฒนาการของราครั้งนี้

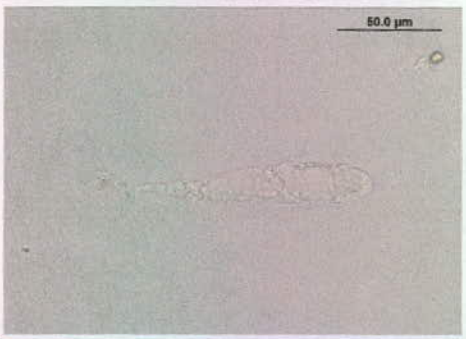
การศึกษาขั้นต่อไปมีการนำสายพันธุ์ของราที่เกี่ยวข้องมาร่วมศึกษาด้วยและนำยีนอื่น เช่น อาร์พีบีวัน (RPB1) และ อีเอฟวันแอลฟ่า (EF1 α) มาช่วยวิเคราะห์ในการศึกษาเปรียบเทียบต่อไป จนได้ข้อมูลที่สมบูรณ์ที่สุด เพื่อหาออเดอร์ (order) ที่ชัดเจนต่อไป



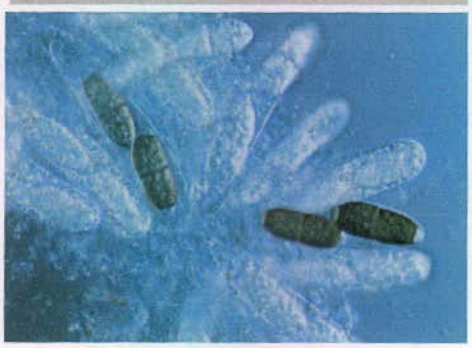
ภาพที่ 1 ตัวอย่าง สปอร์ของรา *Savoryella paucispora* พบจากเศษไม้ที่ถูกย่อยสลายใกล้ชายฝั่งทะเล สปอร์ของรานี้เมื่อโตเจริญเต็มที่จะมีสีเข้มและมีผนังส่วนที่มุมทั้งสองจะมีปุ่มใสมีสีน้ำตาลอ่อน



ภาพที่ 2 ตัวอย่าง *Savoryella paucispora* เป็นราทะเลที่แตกต่างจากราในชนิดเดียวกัน โดยมีการสร้างถุงแอสคัส (ascus) ที่มี แอสโคสปอร์เพียง 2 สปอร์เท่านั้น ราชนิดนี้มีการเจริญเติบโตช้า



ภาพที่ 3 ตัวอย่าง แอสโคสปอร์ของ *Savoryella paucispora* ภายใต้การตรวจสอบกล้องจุลทรรศน์ โดยสังเกตเห็นว่า แอสโคสปอร์ยังเจริญเติบโตไม่เต็มที่ มีสีจางๆราในกลุ่มนี้ส่วนใหญ่จะมีการเจริญเติบโตบนอาหารเลี้ยงช้ามาก



ภาพที่ 4 ถุงแอสคัส ของ *Savoryella paucispora* มีการ เจริญเติบโตแอสโคสปอร์ที่ยังเจริญเติบโตไม่เต็มที่ และเต็มที่ โดยสังเกตเห็นว่าสปอร์ของรานี้เมื่อโตเจริญเต็มที่จะมีสีเข้มขึ้น

ภาพที่ 5 สปอร์ในระยะอาศัยไม่อาศัยเพศ ของรา สกุล *Canalisporium* ราชนิดนี้เป็นราที่พบได้บ่อยจากแหล่งต่าง ๆ ในเขตร้อนชื้น โดยขนาดของสปอร์ ลักษณะรูปร่างของราชนิดนี้มีขนาดค่อนข้างเล็กมาก จัดเป็นราในกลุ่ม Asexual stage ที่อาศัยและพบได้ตามแหล่งน้ำจืดต่างๆ ไม่ว่าจะเป็นลำธาร น้ำตก แม่น้ำ หนองน้ำ